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Europäisches Patentamt
Zweigstelle in Den Haag Recherchenabteilung

European Patent Office
Branch at The Hague Search division

Office européen des brevets
Département à La Haye Division de la recherche

Holdcroft, James Gerald, Dr.
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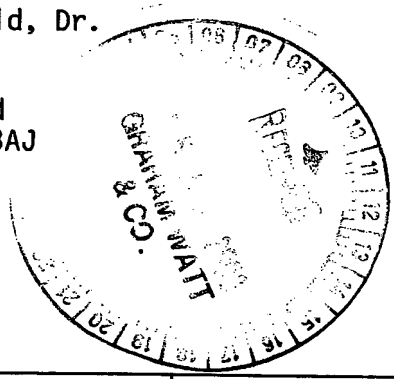

Datum/Date
23. 06. 2003

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°./Patent Nr./Patent No./Brevet n°.
JGH 13973 PCTEP	98949803.5 - -

Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire
CeNeS Pharmaceuticals, Inc.

COMMUNICATION

The European Patent Office herewith transmits

- ☐ the European search report
- ☐ the declaration under Rule 45 EPC
- ☐ the partial European search report under Rule 45 EPC
- ☒ the supplementary European search report concerning the international application under Article 157(2) EPC relating to the above-mentioned European patent application. Copies of the documents cited in the search report are enclosed.

The following specifications given by the applicant have been approved by the Search Division :

☐ Abstract ☐ Title ☐ Figure

☐ The abstract was modified by the Search Division and the definitive text is attached to this communication.

☐ The following figure will be published with the abstract, since the Search Division considers that it better characterises the invention than the one indicated by the applicant.

Figure:

☐ Additional copy(copies) of the documents cited in the European search report.

REFUND OF THE SEARCH FEE

If applicable under Article 10 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

European Patent Office

SUPPLEMENTARY PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 98 94 9803

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	WO 96 15812 A (MARCHIONNI MARK A ;MCBURNEY ROBERT N (US); GWYNNE DAVID I (US); BE) 30 May 1996 (1996-05-30)	1-4	A61K38/18 C07K14/475 A61K48/00 A61P25/00
Y	* see claims 32-35, fig 11, 13.0, 14-16, 24-25, page 18 and page 30 line 1-13 *	1-4	
X	WO 96 30403 A (BERMINGHAM MCDONOGH OLIVIA ;MARCHIONNI MARK A (US); GWYNNE DAVID I) 3 October 1996 (1996-10-03)	1-4	
Y	* see claims 45-46 and 58, fig. 13.0 and page 30 *	1-4	
Y	MARCHIONNI M.A. ET AL.: "Neuregulins as potential drugs for neurological disorders." COLD SPRING HARBOR SYMPOSIA ON QUANTITATIVE BIOLOGY., vol. 61, 1996, pages 459-472, XP009007573 * see the whole document *	1-4	

TECHNICAL FIELDS SEARCHED (Int.Cl.6)

A61K
C07K
A61P

The supplementary search report has been based on the last set of claims valid and available at the start of the search.

INCOMPLETE SEARCH

The Search Division considers that the present application, or some or all of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for the following claims:

Claims searched completely :

Claims searched incompletely :

Claims not searched :

5

Reason for the limitation of the search:

Claim 5 does not contain any additional limiting technical featur as compared to the preceding claims. Defining a product by its desired effect does not limit the scope of a claim in any manner.

Place of search	Date of completion of the search	Examiner
MUNICH	17 March 2003	Merckling, V

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C20)

European Patent Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number
EP 98 94 9803

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1-4 (part)

Use of the neuregulin comprising aminoacid Seq. ID No.2,n or encoded by nucleic acid Seq. ID No.1, for the manufacture of a medicament for treating a mammal suffering from one of the diseases listed in claim 1.

2. Claims: 1-4 (part)

Inventions 2-X : Use of the neuregulin comprising one of the aminoacid sequences as defined in claim 2 (or of a gene encoding such a polypeptide), for the manufacture of a medicament for treating a mammal suffering from one of the diseases listed in claim 1.

European Patent Office

Application Number
EP 98 94 9803

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

☐ Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claim(s):

☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet B

☐ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.

☐ As all searchable claims could be searched without effort justifying an additional fee, the Search Division did not invite payment of any additional fee.

☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:

☒ None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:

1-4 (part.)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 98 94 9803

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

17-03-2003

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9615812 A	30-05-1996	US 6087323 A	11-07-2000
		AU 707599 B	15-07-1999
		AU 4238496 A	17-06-1996
		CA 2204850 A	30-05-1996
		EP 0784488 A	23-07-1997
		JP 10509717 T	22-09-1998
		US 2003040465 A	27-02-2003
WO 9630403 A	03-10-1996	AU 713384 B	02-12-1999
		AU 5376696 A	16-10-1996
		CA 2215330 A	03-10-1996
		EP 0820467 A	28-01-1998
		JP 11509831 T	31-08-1999

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/21349

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/70, 38/00, 38/02, 38/18
US CL :514/2, 12, 44, 903, 907

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 12, 44, 903, 907

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,530,109 A (GOODEARL et al.) 25 June 1996, column 3, line 3 to column 6, line 53 and column 11, line 1 to column 12, line 59.	1-34

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 DECEMBER 1998	Date of mailing of the international search report 05FEB1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer STEPHEN GUCKER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/21349

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 35-36
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, SCISEARCH, EMBASE, BIOSIS, CAPLUS, WPIDS, BIOTECHDS, CONFSCI, LIFESCI
neuregulin#, glia#, heregulin#, GGF#, ischemia#, dementia#, Parkinson#, Huntington#, Alzheimer#, infarct#, amyotrophic, Down#, Korsakoff#, heart#, cardiac, spinal

16

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
47440-PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/21349	08 OCTOBER 1998	14 OCTOBER 1997
International Patent Classification (IPC) or national classification and IPC IPC(7): A61K 31/70, 38/00, 38/02, 38/18 and US Cl.: 514/2, 12, 44, 903, 907		
Applicant CAMBRIDGE NEUROSCIENCE, INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 9 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☒ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
22 APRIL 1999	26 MAY 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer STEPHEN GUCKER
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998)★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US98/21349

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-20 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 21-25 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the drawings:
pages 1-18 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the sequence listing part of the description:
pages 1-43 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[X] contained in the international application in printed form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [X] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

*** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US98/21349

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 35-36

because:

☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination *(specify)*.

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 35-36 are so unclear that no meaningful opinion could be formed *(specify)*.

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for said claims Nos. _.

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US98/21349

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	NONE	YES
	Claims	1-34	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-34	NO
Industrial Applicability (IA)	Claims	1-34	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-34 lack novelty under PCT Article 33(2) as being anticipated by GOODEARL et al. GOODEARL et al. discloses multiple methods of treatment using multiple products that meet all of the claim limitations (see column 3, line 3 to column 6, line 53 for multiple products and uses; also see column 11, line 1 to column 12, line 59 for multiple formulations and methods of treatment of multiple conditions).

Claims 1-34 meet the criteria set out in PCT Article 33(4), because those of skill in the art could use the claimed inventions.

---------- NEW CITATIONS ----------

NONE

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

I. BASIS OF REPORT:

5. (Some) amendments are considered to go beyond the disclosure as filed:
NONE

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CZ	Czech Republic
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EE	Estonia
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FI	Finland
FR	France
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GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
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IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
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LK	Sri Lanka
LR	Liberia
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LT	Lithuania
LU	Luxembourg
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MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
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ZW	Zimbabwe

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WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 31/70, 38/00, 38/02, 38/18

A1

(11) International Publication Number: WO 99/18976

(43) International Publication Date: 22 April 1999 (22.04.99)

(21) International Application Number: PCT/US98/21349

(22) International Filing Date: 8 October 1998 (08.10.98)

(30) Priority Data:
60/062,109 14 October 1997 (14.10.97) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/062,109 (CON)
Filed on 14 October 1997 (14.10.97)

(71) Applicant *(for all designated States except US)*: CAMBRIDGE NEUROSCIENCE, INC. [US/US]; Building 700, One Kendall Square, Cambridge, MA 02139 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MCBURNEY, Robert, N. [US/US]; 20 Leslie Road, Newton, MA 02166 (US). HOLT, William [US/US]; 162 A East Central Street, Natick, MA 01760 (US). GWYNNE, David, I. [US/US]; 77 Grover Street, Beverly, MA 01915 (US). MARCHIONNI, Mark [US/US]; 24 Twin Circle Drive, Arlington, MA 02174 (US).

(74) Agents: CONLIN, David, G. et al.; Dike, Bronstein, Roberts & Cushman, LLP, 130 Water Street, Boston, MA 02109 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: THERAPEUTIC METHODS COMPRISING USE OF A NEUREGULIN

(57) Abstract

The invention provides methods for treatment and/or prophylaxis of certain neurological–related disorders, particularly treatment or prophylaxis of the effects of stroke, brain or spinal cord injury or ischemia, heart attack, optic nerve and retinal injury and ischemia and other acute–type conditions disclosed herein as well as chronic–type conditions, specifically epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, Down's Syndrome, Korsakoff's disease, cerebral palsy and/or age–dependent dementia. The methods of the invention comprise administration of a neuregulin, or fragment or derivative of a neuregulin, or a nucleic acid encoding a neuregulin or a neuregulin fragment or derivative, to a patient suffering from or susceptible to such conditions.

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THERAPEUTIC METHODS COMPRISING USE OF A NEUREGULIN

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to methods for treatment of certain neurological-related injuries and disorders comprising use of a neuregulin, or a fragment or derivative of a neuregulin, or a nucleic acid encoding a neuregulin or neuregulin fragment or derivative.

2. Background

Nerve cell death (degeneration) can cause potentially devastating and irreversible effects for an individual and may occur e.g. as a result of stroke, heart attack or other brain or spinal chord ischemia or trauma. Additionally, neurodegenerative disorders involve nerve cell death (degeneration) such as Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, Down's Syndrome and Korsakoff's disease.

Therapies have been investigated to treat nerve cell degeneration and related disorders, e.g., by limiting the extent of nerve cell death that may otherwise occur to an individual as well as promoting repair, remodeling and reprogramming after stroke or other neuronal injury. See, e.g., F. Seil, *Curr Opin Neuro*, 10:49-51 (1997); N. L. Reddy et al., *J Med Chem*, 37:260-267 (1994); and WO 95/20950.

Certain growth factors have been reported to exhibit neuroprotective properties. In particular, nerve growth factor (NGF) has been evaluated in certain neuroprotective models. See, for example, G. Sinson et al., *J Neurosurg*, 86(3):511-518 (1997); and G. Sinson et al., *J Neurochem*, 65(5):2209-2216 (1995). Osteogenic protein-1 (OP-1) has been evaluated in a rat model of cerebral hypoxia/ischemia for neuroprotective activity. G. Perides, *Neurosci Lett*, 1871):21-24 (1995). Glial cell line-derived neurotrophic factor (GDNF) was reported to exhibit trophic activity on certain populations of central neurons. Y. Wang et al., *J Neurosci*, 17(11):4341-4348 (1997). Small molecules also have been investigated as neuroprotective agents, such

as MK-801. See B. Meldrum , *Cereb Brain Metab Rev*, 2:27-57 (1990); D. Choi, *Cereb Brain Metab Rev*, 2:27-57 (1990).

However, no effective pharmacotherapies are in regular clinical use for ischemia-induced brain injury or other such injuries and disorders. See, for example, Y. Wang et al., *supra*; G. Sinson et al., *J Neurochem*, *J Neurochem*, 65(5):2209 (1995).

It thus would be highly desirable to have new neuroprotective agents, particularly agents to limit the extent or otherwise treat nerve cell death (degeneration) that occur with stroke, heart attack or brain or spinal cord trauma, or to treat Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, Down's Syndrome and Korsakoff's disease. It also would be desirable to have agents that promote repair, remodeling or reprogramming after stroke or other neuronal injury.

SUMMARY OF THE INVENTION

The present invention provides methods for treatment and/or prophylaxis of certain neurological-related disorders, particularly treatment or prophylaxis of the effects of stroke, brain or spinal cord injury or ischemia, heart attack, optic nerve and retinal injury and ischemia and other acute-type conditions disclosed herein as well as chronic-type conditions, specifically epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, Down's Syndrome, Korsakoff's disease, cerebral palsy and/or age-dependent dementia. Methods of the invention also include therapies for promoting repair, remodeling or reprogramming after stroke or other neuronal injury.

The methods of the invention comprise administration of an effective amount of neuregulin, or fragment or derivative of a neuregulin, or a nucleic acid encoding a neuregulin or a neuregulin fragment or derivative (i.e. gene therapy), to a patient suffering from or susceptible to such conditions.

Neuregulins are members of the epidermal growth factor (EGF) superfamily and include glial growth factor (GGF), acetylcholine receptor-inducing activity (ARIA), neu differentiation factor (NDF) and heregulins (HRF). See D. E. Wen et al., *Cell*, 69:559-572 (1992); W.E. Holmes et al., *Science*, 256:1205-1210 (1992); M.A. Marchionni et al., *Nature*, 362:312-318 (1993); and D.L. Falls, *Cell*, 72:801-815

(1993). A variety of neuregulins and fragments and derivatives thereof can be employed in the methods of the invention. For example, suitable agents have been disclosed in U.S. Patent 5,530,109 and PCT/US93/07491. Neuregulins also have been reported in U.S. Patent 5,367,060. Preferred neuregulins include regions shown in FIGS. 1-2 (SEQ ID NOS. 2 and 4), also known as the E sequence. Preferred neuregulins or fragments or derivatives also include those that contain the C, C/D or C/D' sequences as shown in Figures 7, 8 and 9 respectively of the drawings, or those neuregulins or fragments or derivatives that have substantial homology to the peptide sequences shown in Figures 7, 8 or 9, e.g. at least about 70 percent homology, or at least about 80 percent homology, or more preferably at least about 90 or 95 percent homology to the peptide sequences shown in Figures 7, 8 or 9. Preferred nucleic acids and fragments and derivatives for use in the methods of the invention include those nucleic acids that include one or more nucleic acids sequences shown in Figures 7, 8 and 9 of the drawings, or those nucleic acids that that have substantial homology to the nucleic acid sequences shown in Figures 7, 8 or 9, e.g. at least about 70, 80, 90 or 95 percent homology to the nucleic acid sequences shown in Figures 7, 8 or 9. A particularly preferred neuregulin is encoded by DNA obtainable from the clone pGGF2HBS11 (ATCC Deposit No. 75347). Also preferred are neuregulins encoded by DNA obtainable from GGF2BPP5, GGF2BPP2 and GGF2BPP4.

Typical patients that may be treated in accordance with the methods of the invention are persons suffering from brain or spinal cord trauma or ischemia, stroke, heart attack, hypoxia, hypoglycemia, post-surgical neurological deficits, decreased blood flow or nutrient supply to retinal tissue or optic nerve, retinal trauma or ischemia or optic nerve injury. Patients suffering from chronic-type conditions also may be treated in accordance with the invention, specifically subjects suffering from or susceptible to epilepsy, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, Alzheimer's disease, Down's Syndrome, Korsakoff's disease, cerebral palsy and/or age-dependent dementia.

Also, as discussed above, a neuregulin or fragment or derivative thereof or nucleic acid encoding same, may be administered to promote repair, remodeling or reprogramming to a subject that has suffered stroke or other neuronal injury such as traumatic brain or spinal cord injury. In such cases, the therapeutic agent may be

suitably administered to the subject over an extended period following the injury, e.g. at least about 1, 2, 3, 4, 6, 8, 12 or 16 weeks following the injury.

Other aspects of the invention are disclosed *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a nucleotide sequence (SEQ ID NO:1) encoding a preferred neuregulin region (E segment of human GGF) and the amino acid sequence (SEQ ID NO:2) of that preferred region.

FIG. 2 shows a nucleotide sequence (SEQ ID NO:3) encoding a preferred neuregulin region (E segment of bovine GGF) and the amino acid sequence (SEQ ID NO:4) of that preferred region.

FIG. 3 shows nucleotide sequences (SEQ ID NOS:6-7) encoding further neuregulin regions (B segment of human and bovine GGF) and amino acid sequences (SEQ ID NOS:5 and 8) of those regions. Line 1 is the predicted amino acid sequence of bovine B segment, line 2 is a nucleotide sequence of bovine B segment, line 3 is a nucleotide sequence of human B segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of human B segment shown where it differs from the bovine sequence set forth in line 1 of the figure.

FIG. 4 shows nucleotide sequences (SEQ ID NOS:10-11) encoding further neuregulin regions (A segment of human and bovine GGF) and amino acid sequences (SEQ ID NOS:9 and 12) of those regions. Line 1 is the predicted amino acid sequence of bovine A segment, line 2 is a nucleotide sequence of bovine A segment, line 3 is a nucleotide sequence of human A segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of human A segment shown where it differs from the bovine sequence set forth in line 1 of the figure.

FIG. 5 shows a nucleotide sequence (SEQ ID NO:13) encoding a further neuregulin region (A' segment of bovine GGF) and the predicted amino acid sequence (SEQ ID NO:14) of that region.

FIG. 6 shows nucleotide sequences (SEQ ID NOS:16-17) encoding further neuregulin regions (G segment of bovine and human GGF) and amino acid sequences (SEQ ID NOS:15 and 18) of that region. Line 1 is the predicted amino acid sequence of bovine G segment, line 2 is a nucleotide sequence of bovine G segment, line 3 is a

nucleotide sequence of human G segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of human G segment shown where it differs from the bovine sequence set forth in line 1 of the figure.

FIG. 7 shows nucleotide sequences (SEQ ID NOS:20-21) encoding further neuregulin regions (C segment of bovine and human GGF) and amino acid sequences (SEQ ID NOS:19 and 22) of those regions. Line 1 is the predicted amino acid sequence of bovine C segment, line 2 is a nucleotide sequence of bovine C segment, line 3 is a nucleotide sequence of human C segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of human C segment shown where it differs from the bovine sequence set forth in line 1 of the figure.

FIG. 8 shows nucleotide sequences (SEQ ID NOS:24-25) encoding further neuregulin regions (C/D segment of human and bovine GGF) and amino acid sequences (SEQ ID NOS:23 and 26) of those regions. Line 1 is the predicted amino acid sequence of bovine C/D segment, line 2 is a nucleotide sequence of bovine C/D segment, line 3 is a nucleotide sequence of human C/D segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of human C/D segment shown where it differs from the bovine sequence set forth in line 1 of the figure.

FIG. 9 shows nucleotide sequences (SEQ ID NOS:28-29) encoding a further neuregulin region (C/D' segment of the human and bovine GGF) and the amino acid sequence (SEQ ID NO:27) of that region. Line 1 is the predicted amino acid sequence of the C/D' segment, line 2 is a nucleotide sequence of bovine C/D' segment and line 3 is a nucleotide sequence of human C/D' segment (nucleotide base matches are indicated with a vertical line).

FIG. 10 shows nucleotide sequences (SEQ ID NOS:31-32) encoding a further neuregulin region (D segment of the human and bovine GGF) and the amino acid sequence (SEQ ID NO:30) of that region. Line 1 is the predicted amino acid sequence of the D segment, line 2 is a nucleotide sequence of bovine D segment and line 3 is a nucleotide sequence of human D segment (nucleotide base matches are indicated with a vertical line).

FIG. 11 shows nucleotide sequence (SEQ ID NO:34) encoding a further neuregulin region (D' segment of bovine GGF) and the amino acid sequence (SEQ ID NO:33) of that region.

FIGS. 12A-12B show nucleotide sequences (SEQ ID NOS:36-37) encoding further neuregulin regions (H segment of human and bovine GGF) and amino acid sequences (SEQ ID NO:35 and 38) of that region. Line 1 is the predicted amino acid sequence of bovine H segment, line 2 is a nucleotide sequence of bovine H segment, line 3 is a nucleotide sequence of human H segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of human H segment shown where it differs from the bovine sequence set forth in line 1 of the figure.

FIG. 13 shows a nucleotide sequence (SEQ ID NO:40) encoding a further neuregulin region (K segment of bovine GGF) and the amino acid sequence (SEQ ID NO:39) of that region.

FIGS. 14A-14C show nucleotide sequences (SEQ ID NOS:42-43) encoding a further neuregulin region (L segment of bovine and human GGF) and amino acid sequences (SEQ ID NO:41 and 44) of that region. Line 1 is the predicted amino acid sequence of bovine L segment, line 2 is a nucleotide sequence of bovine L segment, line 3 is a nucleotide sequence of human L segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of human L segment shown where it differs from the bovine sequence set forth in line 1 of the figure.

FIG. 15 shows nucleotide sequences (SEQ ID NOS:46-47) encoding further neuregulin regions (F segment of bovine and human GGF) and amino acid sequences (SEQ ID NOS:45 and 48) of that region. Line 1 is the predicted amino acid sequence of bovine F segment, line 2 is a nucleotide sequence of bovine F segment, line 3 is a nucleotide sequence of human F segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of human F segment shown where it differs from the bovine sequence set forth in line 1 of the figure.

FIGS. 16A-16C show the nucleotide sequence (SEQ ID NO:49) and deduced amino acid sequence (SEQ ID NO:50) of GGF2BPP4.

FIGS. 17A-17B show the nucleotide sequence (SEQ ID NO:51) and deduced amino acid sequence (SEQ ID NO:52) of GGF2BPP2.

FIGS. 18A-18B show the nucleotide sequence (SEQ ID NO:53) and deduced amino acid sequence (SEQ ID NO:54) of GGF2BPP5.

DETAILED DESCRIPTION OF THE INVENTION

As discussed above, preferred neuregulins for use in the therapeutic methods of the present invention include those disclosed in U.S. Patent 5,530,109 and PCT/US93/07491, incorporated herein by reference. Particularly preferred neuregulins comprise an amino acid sequence of the following formula:

WYBAZCX

wherein WYBAZCX is composed of amino acid sequences that include one or more sequences shown in FIGS. 1 through 15 (which includes SEQ ID NOS:2, 4, 5, 8, 9, 12, 14, 15, 18, 19, 22, 23, 26, 27, 30, 33, 35, 38, 39, 41, 44, 45 and 48), wherein W comprises the polypeptide segment F, or is absent; wherein Y comprises the polypeptide segment E, or is absent; wherein Z comprises the polypeptide segment G or is absent; and wherein X comprise a polypeptide segment selected from the group consisting of C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/d D'H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL and C/D C/D' D' HKL, and preferably that either

a) at least one of F, Y, B, A, Z, C or X is of bovine origin; or

b) Y comprises the polypeptide segment E; or

c) X comprises the polypeptide segments C/D HKL, C/D D, C/D' HKL, C/D C/D' HKL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HKL, C/D C/D' D'H, C/D C/D' D HL, C/D C/D' D' HKL, C/D'H, C/D C/D' H or C/D C/D' HL.

Particularly preferred neuregulins also include those polypeptides that include the segments FB polypeptides that include the segments FBA' (i.e. the groups F, B and A' as defined herein including in the drawings); polypeptides that include the segments EBA (i.e. the groups E, B and A as defined herein including in the drawings); polypeptides that include the segments EBA' (i.e. the groups E, B and A' as defined herein including in the drawings); A (i.e. the group A as defined herein

including in the drawings); polypeptides that include the segments FEBA (i.e. the groups F, E, B and A as defined herein including in the drawings); polypeptides that include the segments FBA' (i.e. the groups F, B and A' as defined herein including in the drawings); and polypeptides that include the segments FEBA' (i.e. the groups F, E, B and A' as defined herein including in the drawings).

Also preferred are nucleic acids that code for the above preferred polypeptides.

A "fragment" or "derivative" of a neuregulin refers to herein 1) a peptide in which one or more amino acid residues are with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) a peptide in which one or more of the amino acid residues includes a substituent group, or (iii) a peptide in which the mature protein is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol). Thus, a fragment or derivative for use in accordance with the methods of the invention includes a proprotein, which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The polypeptide fragments and derivatives of the invention are of a sufficient length to uniquely identify a region of a neuregulin. Neuregulin fragments thus preferably comprise at least 8 amino acids, usually at least about 12 amino acids, more usually at least about 15 amino acids, still more typically at least about 30 amino acids, even more typically at least about 50 or 70 amino acids. Preferred fragments or derivatives for use in the methods of the invention include those that have at least about 70 percent homology (sequence identity) to any of the preferred sequences mentioned above, more preferably about 80 percent or more homology to any of the preferred sequences mentioned above, still more preferably about 85 to 90 percent or more homology to any of the preferred sequences mentioned above. Sequence identity or homology with respect to a neuregulin as referred to herein is the percentage of amino acid sequences of a neuregulin protein or fragment or derivative thereof that are identical with a specified sequence, after introducing any gaps necessary to achieve the maximum percent homology.

The neuregulin fragments and derivatives for use in the methods of the invention preferably exhibit good activity in standard neuroprotective assays such as

the *in vivo* cerebral ischemia assay of Example 1, which follows. That assay includes the following steps: a) continuous intraventricular infusion of the protein fragment or derivative or vehicle alone to test rats for three days prior to inducing focal ischemic infarcts in right lateral cerebral cortex; and b) twenty-four hours after inducing ischemic infarcts, infarct volume in each test animal is determined by image analysis. Preferably, a protein fragment or derivative of the invention provides at least about a 10% reduction in infarct volume relative to vehicle-treated animals, more preferably about a 20% reduction in infarct volume, still more preferably about a 25% reduction in infarct volume relative to vehicle-treated animals in such an assay. References herein to *in vivo* cerebral ischemia assay are intended to refer to an assay of the above steps a) and b), which are more fully described in Example 1 which follows.

As discussed above, neuregulin nucleic acid fragments and derivatives are also provided for use in the methods of the invention. Those fragments and derivatives typically are of a length sufficient to bind to a sequence of any of the nucleic acid sequences shown in Figures 1-15 of the drawings, including SEQ ID NOS:1, 3, 6, 7, 10, 11, 13, 16, 17, 20, 21, 24, 25, 28, 29, 31, 32, 34, 36, 37, 40, 42 and 43 under the following moderately stringent conditions (referred to herein as "normal stringency" conditions): use of a hybridization buffer comprising 20% formamide in 0.8M saline/0.08M sodium citrate (SSC) buffer at a temperature of 37°C and remaining bound when subject to washing once with that SSC buffer at 37°C.

Preferred neuregulin nucleic acid fragments and derivatives of the invention will bind to a sequence of any of the nucleic acid sequences shown in Figures 1-15 of the drawings, including SEQ ID NOS:1, 3, 6, 7, 10, 11, 13, 16, 17, 20, 21, 24, 25, 28, 29, 31, 32, 34, 36, 37, 40, 42 and 43 under the following highly stringent conditions (referred to herein as "high stringency" conditions): use of a hybridization buffer comprising 20% formamide in 0.9M saline/0.09M sodium citrate (SSC) buffer at a temperature of 42°C and remaining bound when subject to washing twice with that SSC buffer at 42°C.

The neuregulin nucleic acid fragments and derivatives preferably should comprise at least 20 base pairs, more preferably at least about 50 base pairs, and still more preferably a nucleic acid fragment or derivative of the invention comprises at least about 100, 200, 300 or 400 base pairs. In some preferred embodiments, the

nucleic acid fragment or derivative is bound to some moiety which permits ready identification such as a radionucleotide, fluorescent or other chemical identifier.

Isolated neuregulin and peptide fragments or derivatives of the invention are preferably produced by recombinant methods, although suitable neuregulins also can be isolated from various sources. See the procedures disclosed U.S. Patent 5,530,109; U.S. Patent 5,367,060; and PCT/US93/07491, incorporated herein by reference. A wide variety of molecular and biochemical methods are available for generating and expressing neuregulin; see e.g. the procedures disclosed in *Molecular Cloning, A Laboratory Manual* (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor), *Current Protocols in Molecular Biology* (Eds. Aufubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, N.Y. 1992) or other procedures that are otherwise known in the art. For example, neuregulin or fragments or derivatives thereof may be obtained by chemical synthesis, or more preferably by expression in bacteria such as *E coli* and eukaryotes such as yeast, baculovirus, or mammalian cell-based expression systems, etc., depending on the size, nature and quantity of neuregulin or fragment or derivative thereof. More particularly, a recombinant DNA molecule comprising a vector and a DNA segment encoding neuregulin, or a fragment or derivative thereof, can be constructed. Suitable vectors include e.g. baculovirus-derived vectors for expression in insect cells (see Pennock et al., Mol. Cell. Biol., 4:399-406 (1984)), T7-based expression vector for expression in bacteria (see Rosenberg et al., Gene, 56:125-135 (1987)) and the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, J. Biol. Chem., 263:3521-3527 (1988)). The DNA segment can be present in the vector operably linked to regulatory elements, e.g., a promoter (e.g., polyhedron, T7 or metallothionein (Mt-I) promoters), or a leader sequence to provide for secretory expression of the polypeptide. The recombinant DNA molecule containing the DNA coding for a neuregulin or a fragment or derivative thereof can be introduced into appropriate host cells by known methods. Suitable host cells include e.g. prokaryotes such as E. coli, Bacillus subtilus, etc., and eukaryote such as animal cells and yeast strains, e.g., S. cerevisiae. Mammalian cells may be preferred such as J558, NSO, SP2-O or CHO. In general, conventional culturing conditions can be employed. See Sambrook, *supra*. Stable transformed or transfected cell lines can then be selected.

The expressed neuregulin or fragment or derivative thereof then can be isolated and purified by known methods. Typically the culture medium is centrifuged and the supernatant purified by affinity or immunoaffinity chromatography, e.g. Protein-A or Protein-G affinity chromatography or an immunoaffinity protocol comprising use of monoclonal antibodies that bind neuregulins.

Neuregulin nucleic acids used in the methods of the invention are typically isolated, meaning the nucleic acids comprise a sequence joined to a nucleotide other than that which it is joined to on a natural chromosome and usually constitute at least about 0.5%, preferably at least about 2%, and more preferably at least about 5% by weight of total nucleic acid present in a given fraction. A partially pure nucleic acid constitutes at least about 10%, preferably at least about 30%, and more preferably at least about 60% by weight of total nucleic acid present in a given fraction. A pure nucleic acid constitutes at least about 80%, preferably at least about 90%, and more preferably at least about 95% by weight of total nucleic acid present in a given fraction.

As discussed above, the present invention includes methods for treating and preventing certain neurological-related injuries and disorders, comprising the administration of an effective amount of a neuregulin or fragment or derivative thereof, or nucleic acid encoding same, to a subject including a mammal, particularly a human, in need of such treatment.

In particular, the invention provides methods for treatment and/or prophylaxis of nerve cell death (degeneration) resulting from hypoxia, hypoglycemia, brain or spinal cord ischemia, brain or spinal cord trauma, stroke, heart attack or drowning. Typical candidates for treatment include e.g. heart attack, stroke and/or persons suffering from cardiac arrest neurological deficits, brain or spinal cord injury patients, patients undergoing major surgery such as heart surgery where brain ischemia is a potential complication and patients such as divers suffering from decompression sickness due to gas emboli in the blood stream. Candidates for treatment also will include those patients undergoing a surgical procedure involving extra-corporal circulation such as e.g. a bypass procedure.

The invention also provides methods for treatment which comprise administration of a neuregulin or fragment or derivative thereof, or nucleic acid

encoding same, to a patient that is undergoing surgery or other procedure where brain or spinal cord ischemia is a potential risk. For example, carotid endarterectomy is a surgical procedure employed to correct atherosclerosis of the carotid arteries. Major risks associated with the procedure include intraoperative embolization and the danger of hypertension in the brain following increased cerebral blood flow, which may result in aneurysm or hemorrhage. Thus, an effective amount of a neuregulin or fragment or derivative thereof, or nucleic acid encoding same, could be administered pre-operatively or peri-operatively to reduce such risks associated with carotid endarterectomy, or other post-surgical neurological deficits.

The invention also is effective to promote and enhance recovery from acute nerve cell death and neurological conditions. Thus, for example, a neuregulin or fragment or derivative thereof, or nucleic acid encoding same, could be administered to promote repair, remodeling or reprogramming to a patient that has suffered from stroke or other neuronal injury, suitably for an extended period as discussed above. A therapeutic agent of the invention also could be administered post-operatively to promote recovery from any neurological deficits that may have occurred to a patient that has undergone surgery.

The invention further includes methods for prophylaxis against neurological deficits resulting from e.g. coronary artery bypass graft surgery and aortic valve replacement surgery, or other procedure involving extra-corporal circulation. Those methods will comprise administering to a patient undergoing such surgical procedures an effective amount of a neuregulin or fragment or derivative thereof, or nucleic acid encoding same, typically either pre-operatively or peri-operatively.

The invention also provides methods for prophylaxis and treatment against neurological injury for patients undergoing myocardial infarction, a procedure that can result in ischemic insult to the patient. Such methods will comprise administering to a patient undergoing such surgical procedure an effective amount of a neuregulin or fragment or derivative thereof, or nucleic acid encoding same, typically either pre-operatively or peri-operatively.

Also provided are methods for treating or preventing neuropathic pain such as may be experienced by cancer patients, persons having diabetes, amputees and other persons who may experience neuropathic pain. These methods for treatment comprise

administration of an effective amount of a neuregulin or fragment or derivative thereof, or nucleic acid encoding same, to a patient in need of such treatment.

The invention also provides methods for treatment and prophylaxis against retinal ischemia or degeneration and resulting visual loss. For example, a neuregulin or fragment or derivative thereof, can be administered parenterally or by other procedure as described herein to a subject a suffering from or susceptible to ischemic insult that may adversely affect retinal function, e.g., significantly elevated intraocular pressures, diseases such as retinal artery or vein occlusion, diabetes or other ischemic ocular-related diseases. Post-ischemic administration also may limit retinal damage. The invention also includes methods for treating and prophylaxis against decreased blood flow or nutrient supply to retinal tissue or optic nerve, or treatment or prophylaxis against retinal trauma or optic nerve injury. Subjects for treatment according to such therapeutic methods of the invention may be suffering or susceptible to retinal ischemia that is associated with atherosclerosis, venous capillary insufficiency, obstructive arterial or venous retinopathies, senile macular degeneration, cystoid macular edema or glaucoma, or the retinal ischemia may be associated with a tumor or injury to the mammal. Intravitreal injection also may be a preferred administration route to provide more direct treatment to the ischemic retina.

The invention further provides a method of treating Korsakoff's disease, a chronic alcoholism-induced condition, comprising administering to a subject including a mammal, particularly a human, an effective amount of a neuregulin or fragment or derivative thereof, in an amount effective to treat the disease. Compounds of the invention are anticipated to have utility for the attenuation of cell loss, hemorrhages and/or amino acid changes associated with Korsakoff's disease.

The invention further includes methods for treating a person suffering from or susceptible to epilepsy, emesis, narcotic withdrawal symptoms and age-dependent dementia, comprising administering to a subject including a mammal, particularly a human, an effective amount of a neuregulin or fragment or derivative thereof, in an amount effective to treat the condition.

It will be appreciated that in some instances a neuregulin or a fragment or derivative thereof will be preferably administered to a subject rather than a neuregulin nucleic acid, particularly where a patient is suffering from or susceptible to an acute

neurological injury that demands immediate therapy. For example, administration of a neuregulin polypeptide may be preferred to a patient suffering from stroke, heart attack, traumatic brain injury and the like where it is desired to deliver the active therapeutic as quickly as possible.

In the therapeutic methods of the invention, neuregulin peptides and nucleic acids may be suitably administered to a subject such as a mammal, particularly a human, by any of a number of routes including parenteral (including subcutaneous, intramuscular, intravenous and intradermal), oral, rectal, nasal, vaginal and optical (including buccal and sublingual) administration. A neuregulin protein or nucleic acid or fragment or derivative thereof may be administered to a subject alone or as part of a pharmaceutical composition, comprising the peptide or nucleic acid together with one or more acceptable carriers and optionally other therapeutic ingredients. The carriers should be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

Nucleic acids encoding a neuregulin or a neuregulin fragment or derivative can be administered to a patient by generally known gene therapy procedures. See, for example, WO 90/11092 and WO 93/00051. Thus, for instance, the nucleic acids may be introduced into target cells by any method which will result in the uptake and expression of the nucleic acid by the target cells. These methods can include vectors, liposomes, naked DNA, adjuvant-assisted DNA, catheters, etc. Preferably, the administered nucleic acid codes for an appropriate secretory sequence to promote expression upon administration. Suitable vectors for administering a nucleic acid in accordance with the invention include chemical conjugates such as described in WO 93/04701, which has targeting moiety (e.g. a ligand to a cellular surface receptor), and a nucleic acid binding moiety (e.g. polylysine), viral vector (e.g. a DNA or RNA viral vector), fusion proteins such as described in PCT/US 95/02140 (WO 95/22618) which is a fusion protein containing a target moiety (e.g. an antibody specific for a target cell) and a nucleic acid binding moiety (e.g. a protamine), plasmids, phage, etc. The vectors can be chromosomal, non-chromosomal or synthetic.

Preferred vectors include viral vectors, fusion proteins and chemical conjugates. Retroviral vectors include moloney murine leukemia viruses. DNA viral vectors are preferred. These vectors include pox vectors such as orthopox or avipox

vectors, herpes virus vectors such as a herpes simplex I virus (HSV) vector [A.I. Geller et al., *J. Neurochem,* 64:487 (1995); F. Lim et al., in *DNA Cloning: Mammalian Systems*, D. Glover, Ed. (Oxford Univ. Press, Oxford England) (1995); A.I. Geller et al., *Proc Natl. Acad. Sci.* U.S.A.:90 7603 (1993); A.I. Geller et al., *Proc Natl. Acad. Sci USA*, 87:1149 (1990)], Adenovirus Vectors [LeGal LaSalle et al., *Science*, 259:988 (1993); Davidson, et al., Nat. Genet., 3:219 (1993); Yang et al., *J. Virol.*, 69:2004 (1995)] and Adeno-associated Virus Vectors [Kaplitt, M.G., et al., *Nat. Genet.*, 8:148 (1994)].

Pox viral vectors introduce the gene into the cell cytoplasm. Avipox virus vectors result in only a short-term expression of the nucleic acid. Adenovirus vectors, adeno-associated virus vectors and herpes simplex virus (HSV) vectors are preferred for introducing the nucleic acid into neural cells. The adenovirus vector results in a shorter term expression (about 2 months) than adeno-associated virus (about 4 months), which in turn is shorter than HSV vectors. The particular vector chosen will depend upon the target cell and the specific condition being treated. The introduction can be by standard techniques, e.g. infection, transfection, transduction or transformation. Examples of modes of gene transfer include e.g., naked DNA, $Ca_3(PO_4)_2$ precipitation, DEAE dextran, electroporation, protoplast fusion, lipofecton, cell microinjection, and viral vectors.

A vector can be employed to target essentially any desired target cell. For example, stereotaxic injection can be used to direct the vectors (e.g. adenovirus, HSV) to a desired location. Additionally, the particles can be delivered by intracerebroventricular (icv) infusion using a minipump infusion system, such as a SynchroMed Infusion System. A method based on bulk flow, termed convection, has also proven effective at delivering large molecules to extended areas of the brain and may be useful in delivering the vector to the target cell (Bobo et al., *Proc. Natl. Acad. Sci. USA*, 91:2076-2080 (1994); Morrison et al., *Am. J. Physiol.*, 266:292-305 (1994)). Other methods that can be used include catheters, intravenous, parenteral, intraperitoneal and subcutaneous injection, and oral or other known routes of administration.

Parenteral formulations for administration of a neuregulin or a fragment or derivative thereof may be in the form of liquid solutions or suspensions; for oral

administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found in, for example, "Remington's Pharmaceutical Sciences". Formulations for parenteral administration may, for example, contain as excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes, biocompatible, biodegradable lactide polymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the present factors. Other potentially useful parenteral delivery systems for a neuregulin or fragments or derivatives thereof include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal administration.

The concentration of a neuregulin or a fragment or derivative thereof, or nucleic acid encoding such polypeptides, administered to a particular subject will vary depending upon a number of issues, including the condition being treated, the mode and site of administration, the age, weight sex and general health of the subject, and other such factors that are recognized by those skilled in the art. Optimal administration rates for a given protocol of administration can be readily determined by those skilled in the art.

All documents mentioned herein are incorporated herein by reference in their entirety. The invention is further illustrated by the following non-limiting Examples.

Example 1 -- In vivo neuroprotection assay

Neuregulins and neuregulin fragments and derivatives can be assessed for neuroprotective efficacy pursuant to the following assay.

Mature male Long-Evans rats (Charles River, 250-350g) are allowed food and water *ad libitum*. Animals are anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and placed in a stereotaxic head holder (David Kopf Instruments, Tujunga, CA). The

dorsal surface of the skull is exposed by midline incision, and a small burr hole (2 mm diameter) is drilled over the right lateral ventricle, 1.6 mm lateral and 0.9 mm posterior to bregma. A stainless steel cannula (I.D. 0.020", O.D. 0.028", 2 cm long) is then inserted stereotaxically into the ventricle to a depth of 4.4 mm beneath the surface of the skull. The tubing is suitably bent at a 90° angle 1-1.6 cm from its tip and connected to polyethylene tubing (I.D. 0.76 mm, O.D. 1.22 mm, 10 cm long) that is connected (by glue) to a mini-osmotic pump (Alzet 1007D, 100 µl fill volume, pump rate = 0.5 µl/hr; Alza Corp., Palo Alto, CA) implanted subcutaneously in the back. The cannula can be suitably fixed to the skull by orthodontic resin (L.D. Culk Co., Milford, DE) bonded to two small machine screws (1/8" stainless steel slotted) inserted in the skull. The pump, tubing, and cannula are primed before insertion with infusate solutions; a 3-0 nylon suture is inserted into the cannula during implantation to prevent obstruction by brain tissue. The wound is closed with 3-0 silk suture and cefazolin (10 mg, i.m.) is administered. After surgery animals are suitably kept in individual cages and fed soft food.

Pumps are filled with vehicle alone (containing 127 mM NaCl, 2.6 mM KCl, 1.2 mM $CaCl_2$, 0.9 mM $MgCl_2$, 4.14 mM HEPES, 3 mM glycerin, 0.001% bovine serum albumin [BSA], and 0.01% fast green), or vehicle neuregulin or fragment or derivative thereof (100 µgm/ml). Heparin can be suitably used at relatively low doses, e.g. about 0.8 units/kg/day which is approximately 250-500 times less than a standard anticoagulant dose.

Three days after cannula implantation, animals are reanesthetized with 2% halothane and given atropine (0.15 mg/kg, i.p.). Animals are then intubated and connected to a ventilator (SAR-830; CWE Inc., Ardmore, PA) delivering 1% halothane/70% nitrous oxide in oxygen. The right femoral artery and vein are cannulated for monitoring of mean arterial blood pressure (MABP; Gould RS3200 Blood Pressure Monitor, Gould Inc., Valley View, OH), and blood sampling. Animals are then paralyzed with pancuronium bromide (0.5 mg/kg, i.v.). Arterial blood gasses (Corning 178 Blood Gas Analyzer, Ciba Corning Diagnostic Corp., Medford, MA), blood glucose (Accu-Check Blood Glucose Analyzer, Boehringer Mannheim, Indianapolis, IN), and hematocrit are measured at least twice during surgery and the immediate post-operative period. The stroke volume and rate of the

ventilator are adjusted to maintain PaO_2 between 100-200 mm Hg and $PaCO_2$ between 30-40 mm Hg. Core body temperature may be monitored by rectal thermocouple (e.g. Model 73ATA, Yellow Springs Instrument Co., Yellow Springs, OH) and maintained between 36-37°C with a homeothermic blanket control unit (Harvard Bioscience, South Natick, MA).

Focal ischemic infarcts are made in the right lateral cerebral cortex in the territory of the middle cerebral artery (MCA) by the method of Chen, et al., *Stroke*, 17:738-743 (1986). Both common carotid arteries are exposed by midline anterior cervical incision. The animal is placed in a lateral position and a 1 cm skin incision is then made at the midpoint between the right lateral canthus and the anterior pinna. The temporal muscle is retracted, and a small (3 mm diameter) craniectomy is made at the junction of the zygoma and squamosal bone using a dental drill cooled with saline. Using a dissecting microscope, the dura can be opened with fine forceps, and the right MCA can be ligated with two 10-0 monofilament nylon ties just above the rhinal fissure and transected between the ties. Both common carotid arteries then can be occluded by microaneurysm clips for 45 minutes. After removal of the clips, return of flow is visualized in the arteries. Anesthesia is maintained for 15 minutes, and animals are returned to individual cages and fed soft food after surgery.

Twenty four hours after cerebral infarction, animals are again weighed, and then sacrificed by rapid decapitation. Brains are removed, inspected visually for the anatomy of the middle cerebral artery as well as for signs of hemorrhage or infection, immersed in cold saline for 10 minutes, and sectioned into six standard coronal slices (each 2 mm thick) using a rodent brain matrix slicer (Systems, Warren, MI). Brains are also examined visually for the presence of dye (fast green) in the cerebral ventricles. Slices are placed in the vital dye 2,3,5-triphenyl tetrazolium chloride (TTC, 2%; Chemical Dynamics Co., NH) at 37°C in the dark for 30 minutes, followed by 10% formalin at room temperature overnight. The outline of right and left cerebral hemispheres as well as that of infarcted tissue, clearly visualizable by lack of TTC staining (Chen et al., *Stroke*, 17:738-743 (1986)), is outlined on the posterior surface of each slice using an image analyzer (MTI videocamera and Sony video monitor connected to a Bioquant IV Image Analysis System run on an EVEREX computer). Infarct volume is calculated as the sum of infarcted area per slice multiplied by slice

thickness. Both the surgeon and image analyzer operator are blinded to the treatment given each animal.

Volumes of infarcts among vehicle vs. neuregulin-treated animals can be compared by unpaired, two-tailed t-tests for each experiment, and by two-way analysis of variance (ANOVA; Exp. X Treatment) for combined data. A subsequent slice-by-slice analysis of infarct area among pooled neuregulin- vs. vehicle-treated animals is suitably done by repeated measures two-way ANOVA (Treatment X Slice). Other anatomical and physiological measurements are compared among GDF-1- vs. vehicle-treated animals by unpaired, two-tailed t-tests using the Bonferroni correction for multiple pairwise comparisons.

Example 2 -- In vivo behavioral assays

For behavioral outcome studies, such as to assess recovery, repair and remodeling promoted by administration of a neuregulin or fragment or derivative thereof, or nucleic acid encoding same, a number of assays can be employed such as those described in G. Sinson et al., *J Neurochem*, 65(5):2209-2214 (1995); T.K. McIntosh et al., *Neuroscience*, 28:233-244 (1989); and T.K. McIntosh et al., *J Neurotrauma*, 10:373-384 (1993).

Briefly, one suitable behavioral assay as described in G. Sinson et al., *supra*, entails that test animals (male Sprague-Dawley rats) receive preinjury training in a Morris Water Maze, a circular tank 1 m in diameter that is filled with 18°C water. The water surface is made opaque with a covering of Styrofoam pieces. During training of the animals a submerged platform is present in the maze. Each test animal undergoes 20 training trials over a two day period during which they learn to locate the platform using external visual cues. Immediately following the last training trial, animals are anesthetized and subjected to a lateral (parasagittal) fluid-percussion (FP) brain injury. Briefly, a 5-mm craniectomy is performed over the left parietal cortex, midway between lamda and bregma. A hollow Leur-loc fitting is cemented to the craniectomy site. The injury is delivered after attaching the FP device. The injury should be of moderate severity (2.1-2.3 atm). After injury, the Leur-loc is removed, and the skin is sutured. Normothermia is maintained with warming pads until the animals being to ambulate.

At 72 hours, 1 week or 2 weeks after injury, animals are assessed for their ability to remember the learned task of locating the platform in the MWM. For this evaluation the platform is removed from the maze, and the animal's swimming pattern is suitably recorded with a computerized video system for 1 minute. The maze is separated in zones that are weighed according to the proximity to the platform's location. A memory score is generated by multiplying the weighted numbers by the time the animal spends in each zone and then adding the products.

Animals surviving for 1 or 2 weeks also can undergo evaluation of neurologic motor function. Briefly, one suitable assay provides that animals are scored from 0 (severely impaired) to 4 (normal) for each of the following: (1) left and (2) right forelimb during suspension by the tail; (3) left and (4) right hindlimb flexion when the forelimbs remain on a surface and the hindlimbs are lifted up and back by the tail; the ability to resist lateral pulsion to the (5) left and (6) right; and the ability to stand on an inclined plane in the (7) left, (8) right, and (9) vertical positions. Scores are combined for each of the tests (1) through (9). The observer for the tests should be blinded to the animal's previous treatment.

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

What is claimed is:

1. A method of treating a mammal suffering from or susceptible to stroke, brain or spinal cord injury or ischemia, or heart attack, comprising administering to the mammal a therapeutically effective amount of a neuregulin, or fragment or derivative of a neuregulin, or a nucleic acid encoding a neuregulin or a fragment or derivative of a neuregulin.

2. A method of treating a mammal suffering from or susceptible to optic nerve injury or retinal injury or ischemia, comprising administering to the mammal a therapeutically effective amount of a neuregulin, or fragment or derivative of a neuregulin, or a nucleic acid encoding a neuregulin or a fragment or derivative of a neuregulin.

3. A method of treating a mammal suffering from or susceptible to effects of post-surgical neurological deficits, hypoxia or hypoglycemia, comprising administering to the mammal a therapeutically effective amount of a neuregulin, or fragment or derivative of a neuregulin, or a nucleic acid encoding a neuregulin or a fragment or derivative of a neuregulin.

4. A method of treating a mammal suffering from or susceptible to epilepsy, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, Alzheimer's disease, Down's Syndrome, Korsakoff's disease, or age-dependent dementia, comprising administering to the mammal a therapeutically effective amount of a neuregulin, or fragment or derivative of a neuregulin, or a nucleic acid encoding a neuregulin or a fragment or derivative of a neuregulin.

5. The method of claim 1 wherein the neuregulin, or fragment or derivative of a neuregulin, or a nucleic acid encoding a neuregulin or a fragment or derivative of a neuregulin is administered after the subject has suffered a stroke, brain or spinal cord injury or ischemia, or heart attack.

6. The method of claim 5 wherein the neuregulin, or fragment or derivative of a neuregulin, or a nucleic acid encoding a neuregulin or a fragment or derivative of a neuregulin is administered to the subject for at least about two weeks after the subject has suffered a stroke, brain or spinal cord injury or ischemia, or heart attack.

7. A method of any one of claims 1-6 wherein a neuregulin or a fragment or derivative thereof is administered to the mammal.

8. A method of claim 7 wherein the neuregulin or fragment or derivative thereof comprises an amino acid sequence of the following formula:

WYBAZCX

wherein WYBAZCX is composed of amino acid sequences that include one or more sequences shown in FIGS. 1 through 15 (which includes SEQ ID NOS:2, 4, 5, 8, 9, 12, 14, 15, 18, 19, 22, 23, 26, 27, 30, 33, 35, 38, 39, 41, 44, 45 and 48), wherein W comprises the polypeptide segment F, or is absent; wherein Y comprises the polypeptide segment E, or is absent; wherein Z comprises the polypeptide segment G or is absent; and wherein X comprise a polypeptide segment selected from the group consisting of C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/d D'H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL and C/D C/D' D' HKL, and preferably that either

a) at least one of F, Y, B, A, Z, C or X is of bovine origin; or

b) Y comprises the polypeptide segment E; or

c) X comprises the polypeptide segments C/D HKL, C/D D, C/D' HKL, C/D C/D' HKL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HKL, C/D C/D' D'H, C/D C/D' D HL, C/D C/D' D' HKL, C/D'H, C/D C/D' H or C/D C/D' HL.

9. The method of claim 7 wherein the neuregulin or fragment or derivative thereof a) has at least one of F, Y, B, A, Z, C or X is of bovine origin; or b) Y comprises the polypeptide segment E; or c) X comprises the polypeptide segments C/D HKL, C/D D, C/D' HKL, C/D C/D' HKL, C/D C/D' D, C/D D H, C/D D' HL, C/D D' HKL, C/D C/D' D' H, C/D C/D' D HL, C/D C/D' D' HKL, C/D'H, C/D C/D' H or C/D C/D' HL.

10. The method of claim 7 wherein the neuregulin or fragment or derivative thereof comprises FBA polypeptide segments, FEBA polypeptides segments, EBA polypeptide segments, EBA' polypeptide segments or FEBA' polypeptide segments.

11. A method of claim 7 wherein the neuregulin is encoded by a nucleic acid that comprises one of SEQ ID NOS:49, 51 and 53.

12. A method of claim 7 wherein the neuregulin or fragment or derivative thereof is encoded by a nucleic acid that comprises a sequence that has at least about 70% sequence identity to one of SEQ ID NOS:49, 51 and 53.

13. A method of claim 7 wherein the neuregulin or fragment or derivative thereof is encoded by a sequence that hybridizes to one of SEQ ID NOS:49, 51 or 53 under normal stringency conditions.

14. A method of claim 7 wherein the neuregulin or fragment or derivative thereof is encoded by a sequence that hybridizes to one of SEQ ID NOS:49, 51 or 53 under high stringency conditions.

15. A method of claim 7 wherein the neuregulin or fragment or derivative has at least about 70% sequence identity to SEQ ID NOS:50, 52 or 54.

16. A method of claim 7 wherein the neuregulin or fragment or derivative thereof is encoded by a nucleic acid that comprises a sequence that has at least about 70% sequence identity to one of SEQ ID NO:20 (Figure 7); SEQ ID NO:21 (Figure 7); SEQ ID NO:24 (Figure 8); SEQ ID NO:25 (Figure 8); SEQ ID NO:28 (Figure 9); or SEQ ID NO:29 (Figure 9).

17. A method of claim 7 wherein the neuregulin or fragment or derivative thereof is encoded by a sequence that hybridizes to one of SEQ ID NO:20 (Figure 7); SEQ ID NO:21 (Figure 7); SEQ ID NO:24 (Figure 8); SEQ ID NO:25 (Figure 8); SEQ ID NO:28 (Figure 9); or SEQ ID NO:29 (Figure 9) under normal stringency conditions.

18. A method of claim 7 wherein the neuregulin or fragment or derivative comprises a sequence that has at least about 70% sequence identity to any of the peptide sequences shown in Figures 7, 8 or 9 of the drawings.

19. A method of claim 7 where the neuregulin or fragment or derivative comprises a sequence that has at least about 80 percent homology to any of the peptide sequences shown in Figures 7, 8 or 9.

20. A method of claim 7 where the neuregulin or fragment or derivative comprises a sequence that has at least about 90 percent homology to any of the peptide sequences shown in Figures 7, 8 or 9.

21. A method of claim 7 wherein the neuregulin or fragment or derivative comprises a sequence that has at least about 95 percent homology to any of the peptide sequences shown in Figures 7, 8 or 9.

22. A method of claim 7 wherein the neuregulin or fragment or derivative comprises a sequence that is shown in Figures 7, 8 or 9.

23. A method of any one of claims 1-6 wherein a nucleic acid encoding a neuregulin or a fragment or derivative thereof is administered to the mammal.

24. A method of claim 23 wherein the nucleic acid is SEQ ID NO:49, 51 or 53, or the complement thereof.

25. A method of claim 23 wherein the nucleic or fragment or derivative thereof encodes a neuregulin or neuregulin fragment or derivative that comprises an amino acid sequence of the following formula:

WYBAZCX

wherein WYBAZCX is composed of amino acid sequences that include one or more sequences shown in FIGS. 1 through 15 (which includes SEQ ID NOS:2, 4, 5, 8, 9, 12, 14, 15, 18, 19, 22, 23, 26, 27, 30, 33, 35, 38, 39, 41, 44, 45 and 48), wherein W comprises the polypeptide segment F, or is absent, wherein Y comprises the polypeptide segment E, or is absent; wherein Z comprises the polypeptide segment G or is absent; and wherein X comprise a polypeptide segment selected from the group consisting of C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL and C/D C/D' D' HKL.

26. The method of claim 25 wherein the neuregulin or neuregulin fragment or derivative a) has at least one of F, Y, B, A, Z, C or X is of bovine origin; or b) Y comprises the polypeptide segment E; or c) X comprises the polypeptide segments C/D HKL, C/D D, C/D' HKL, C/D C/D' HKL, C/D C/D' D, C/D D H, C/D D' HL, C/D D' HKL, C/D C/D' D' H, C/D C/D' D HL, C/D C/D' D' HKL, C/D'H, C/D C/D' H or C/D C/D' HL.

27. The method of claim 25 wherein the neuregulin or neuregulin fragment or derivative comprises FBA polypeptide segments, FEBA polypeptides segments,

EBA polypeptide segments, EBA' polypeptide segments or FEBA' polypeptide segments.

28. A method of claim 23 wherein the nucleic acid comprises a sequence that hybridizes to SEQ ID NO:20 (Figure 7); SEQ ID NO:21 (Figure 7); SEQ ID NO:24 (Figure 8); SEQ ID NO:25 (Figure 8); SEQ ID NO:28 (Figure 9); or SEQ ID NO:29 (Figure 9) under normal stringency conditions.

29. A method of claim 23 wherein the nucleic acid comprises a sequence that hybridizes to SEQ ID NO:SEQ ID NO:20 (Figure 7); SEQ ID NO:21 (Figure 7); SEQ ID NO:24 (Figure 8); SEQ ID NO:25 (Figure 8); SEQ ID NO:28 (Figure 9); or SEQ ID NO:29 (Figure 9) under high stringency conditions.

30. A method of claim 23 wherein the nucleic acid comprises a sequence that has at least about 70 percent homology to any of the nucleic acid sequences shown in Figures 7, 8 or 9.

31. A method of claim 23 wherein the nucleic acid comprises a sequence that has at least about 80 percent homology to any of the nucleic acid sequences shown in Figures 7, 8 or 9.

32. A method of claim 23 wherein the nucleic acid comprises a sequence that has at least about 90 percent homology to any of the nucleic acid sequences shown in Figures 7, 8 or 9.

33. A method of claim 23 wherein the nucleic acid comprises a sequence that has at least about 95 percent homology to any of the nucleic acid sequences shown in Figures 7, 8 or 9.

34. A method of claim 23 wherein the nucleic acid comprises a sequence shown in Figures 7, 8 or 9.

35. A method of any one of claims 1-34 wherein the administered neuregulin fragment or derivative, or the administered nucleic acid encodes a neuregulin fragment or derivative exhibits at least about a 10% reduction in infarct volume in an *in vivo* cerebral ischemia assay.

36. A method of any one of claims 1-35 wherein the mammal is a human.

FIG. 1A
HUMAN SEGMENT E: (SEQ ID NOS:1-2)

```
ATG AGA TGG CGA CGC GCC CCG CGC CGC TCC GGG CGT CCC GGC CCC CGG          48
Met Arg Trp Arg Arg Ala Pro Arg Arg Ser Gly Arg Pro Gly Pro Arg
1               5                  10                  15

GCC CAG CGC CCC GGC TCC GCC GCC CGC TCG TCG CCG CCG CTG CCG CTG          96
Ala Gln Arg Pro Gly Ser Ala Ala Arg Ser Ser Pro Pro Leu Pro Leu
            20                  25                  30

CTG CCA CTA CTG CTG CTG CTG GGG ACC GCG GCC CTG GCG CCG GGG GCG         144
Leu Pro Leu Leu Leu Leu Leu Gly Thr Ala Ala Leu Ala Pro Gly Ala
        35                  40                  45

GCG GCC GGC AAC GAG GCG GCT CCC GCG GGG GCC TCG GTG TGC TAC TCG         192
Ala Ala Gly Asn Glu Ala Ala Pro Ala Gly Ala Ser Val Cys Tyr Ser
    50                  55                  60

TCC CCG CCC AGC GTG GGA TCG GTG CAG GAG CTA GCT CAG CGC GCC GCG         240
Ser Pro Pro Ser Val Gly Ser Val Gln Glu Leu Ala Gln Arg Ala Ala
65                  70                  75                  80

GTG GTG ATC GAG GGA AAG GTG CAC CCG CAG CGG CGG CAG CAG GGG GCA         288
Val Val Ile Glu Gly Lys Val His Pro Gln Arg Arg Gln Gln Gly Ala
                85                  90                  95

CTC GAC AGG AAG GCG GCG GCG GCG GCG GGC GAG GCA GGG GCG TGG GGC         336
Leu Asp Arg Lys Ala Ala Ala Ala Ala Gly Glu Ala Gly Ala Trp Gly
            100                 105                 110

GGC GAT CGC GAG CCG CCA GCC GCG GGC CCA CGG GCG CTG GGG CCG CCC         384
Gly Asp Arg Glu Pro Pro Ala Ala Gly Pro Arg Ala Leu Gly Pro Pro
        115                 120                 125

GCC GAG GAG CCG CTG CTC GCC GCC AAC GGG ACC GTG CCC TCT TGG CCC         432
Ala Glu Glu Pro Leu Leu Ala Ala Asn Gly Thr Val Pro Ser Trp Pro
    130                 135                 140

ACC GCC CCG GTG CCC AGC GCC GGC GAG CCC GGG GAG GAG GCG CCC TAT         480
Thr Ala Pro Val Pro Ser Ala Gly Glu Pro Gly Glu Glu Ala Pro Tyr
145                 150                 155                 160

CTG GTG AAG GTG CAC CAG GTG TGG GCG GTG AAA GCC GGG GGC TTG AAG         528
Leu Val Lys Val His Gln Val Trp Ala Val Lys Ala Gly Gly Leu Lys
                165                 170                 175

AAG GAC TCG CTG CTC ACC GTG CGC CTG GGG ACC TGG GGC CAC CCC GCC         576
Lys Asp Ser Leu Leu Thr Val Arg Leu Gly Thr Trp Gly His Pro Ala
            180                 185                 190
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FIG. 1B

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TTC CCC TCC TGC GGG AGG CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC          624
Phe Pro Ser Cys Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe
        195                 200                 205

ATG GAG CCC GAC GCC AAC AGC ACC AGC CGC GCG CCG GCC GCC TTC CGA          672
Met Glu Pro Asp Ala Asn Ser Thr Ser Arg Ala Pro Ala Ala Phe Arg
    210                 215                 220

GCC TCT TTC CCC CCT CTG GAG ACG GGC CGG AAC CTC AAG AAG GAG GTC          720
Ala Ser Phe Pro Pro Leu Glu Thr Gly Arg Asn Leu Lys Lys Glu Val
225                 230                 235                 240

AGC CGG GTG CTG TGC AAG CGG TGC G                                        745
Ser Arg Val Leu Cys Lys Arg Cys
                245
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FIG.2
SEGMENT E: (SEQ ID NOS:3-4)

```
CC CAT CAA GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG          47
   His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser
     1               5                  10                  15

CTG CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC GCC TTC CCC TCC          95
Leu Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser
                20                  25                  30

TGC GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC         143
Cys Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro
            35                  40                  45

GAG GCC AAC AGC AGC GGC GGG CCC GGC CGC CTT CCG AGC CTC CTT CCC         191
Glu Ala Asn Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro
        50                  55                  60

CCC TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT         239
Pro Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala
    65                  70                  75

GTG CAA CGG TGC G                                                   252
Val Gln Arg Cys
 80
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FIG. 3
SEGMENT B: (SEQ ID NOS:5-8)

```
Leu Pro Pro Arg Leu Lys Glu His Lys Ser Gln Glu Ser Val Ala Gly
CCT TGC CTC CCC GCT TGA AAG AGA TGA AGA GTC AGG AGT CTG TGG CAG          48
||| ||| ||| ||| | | ||| ||| ||| ||| | | | ||| | | | | ||| |||
CCT TGC CTC CCC GAT TGA AAG AGA TGA AAA GCC AGG AAT CGG CTG CAG
            Q                                       A

Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser Ser Leu
GTT CCA AAC TAG TGC TTC GGT GCG AGA CCA GTT CTG AAT ACT CCT CTC          96
||| ||| ||| ||| | | ||| ||| | | | | ||| ||| ||| ||| ||| ||| |||
GTT CCA AAC TAG TCC TTC GGT GTG AAA CCA GTT CTG AAT ACT CCT CTC

Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser Arg Lys Asn Lys
TCA AGT TCA AGT GGT TCA AGA ATG GGA GTG AAT TAA GCC GAA AGA ACA         144
|||  | ||| ||| ||| ||| ||| ||| ||| ||  ||| | |  | ||| | | |||
TCA GAT TCA AGT GGT TCA AGA ATG GGA ATG AAT TGA ATC GAA AAA ACA
R                               N           N

Pro Gly Asn Ile Lys Ile Gln Lys Arg Pro Gly
AAC CAC AAA ACA TCA AGA TAC AGA AAA GGC CGG G                        178
||| ||| ||| | | ||| ||| ||| | | ||| ||  | | |
AAC CAC AAA ATA TCA AGA TAC AAA AAA AGC CAG G
                                    K
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FIG 4
SEGMENT A: (SEQ ID NOS:9-12)

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  Lys Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly
G AAG TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA          46
  ||| ||| ||| ||| ||| ||| | | ||| || ||| ||| ||| ||| ||| |||
G AAG TCA GAA CTT CGC ATT AAC AAA GCA TCA CTG GCT GAT TCT GGA
                        N

Glu Tyr Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser
GAA TAT ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT          94
|| ||| ||| ||| ||| ||| ||| ||| ||| || ||| ||| ||| ||| ||| |||
GAG TAT ATG TGC AAA GTG ATC AGC AAA TTA GGA AAT GAC AGT GCC TCT

Ala Asn Ile Thr Ile Val Glu Ser Asn Ala
GCC AAC ATC ACC ATT GTG GAG TCA AAC G                                 122
||| ||  ||| ||| ||  ||| ||  ||| ||| |
GCC AAT ATC ACC ATC GTG GAA TCA AAC G
```

FIG.5
SEGMENT A': (SEQ ID NOS:13-14)

```
TCTAAAACTA CAGAGACTGT ATTTTCATGA TCATCATAGT TCTGTGAAAT ATACTTAAAC      60

CGCTTTGGTC CTGATCTTGT AGG AAG TCA GAA CTT CGC ATT AGC AAA GCG         110
                          Lys Ser Glu Leu Arg Ile Ser Lys Ala
                            1               5

TCA CTG GCT GAT TCT GGA GAA TAT ATG TGC AAA GTG ATC AGC AAA CTA       158
Ser Leu Ala Asp Ser Gly Glu Tyr Met Cys Lys Val Ile Ser Lys Leu
 10                  15                  20                  25

GGA AAT GAC AGT GCC TCT GCC AAC ATC ACC ATT GTG GAG TCA AAC GGT       206
Gly Asn Asp Ser Ala Ser Ala Asn Ile Thr Ile Val Glu Ser Asn Gly
                 30                  35                  40

AAG AGA TGC CTA CTG CGT GCT ATT TCT CAG TCT CTA AGA GGA GTG ATC       254
Lys Arg Cys Leu Leu Arg Ala Ile Ser Gln Ser Leu Arg Gly Val Ile
            45                  50                  55

AAG GTA TGT GGT CAC ACT TGAATCACGC AGGTGTGTGA AATCTCATTG              302
Lys Val Cys Gly His Thr
        60

TGAACAAATA AAAATCATGA AAGGAAAACT CTATGTTTGA AATATCTTAT GGGTCCTCCT     362

GTAAAGCTCT TCACTCCATA AGGTGAAATA GACCTGAAAT ATATATAGAT TATTT          417
```

FIG. 6
SEGMENT G: (SEQ ID NOS:15-18)

```
Glu Ile Thr Thr Gly Met Pro Ala Ser Thr Glu Thr Ala Tyr Val Ser
 AG ATC ACC ACT GGC ATG CCA GCC TCA ACT GAG ACA GCG TAT GTG TCT      47
 AG ATC ATC ACT GGT ATG CCA GCC TCA ACT GAA GGA GCA TAT GTG TCT
           I                                 G

Ser Glu Ser Pro Ile Arg Ile Ser Val Ser Thr Glu Gly Thr Asn Thr
TCA GAG TCT CCC ATT AGA ATA TCA GTA TCA ACA GAA GGA ACA AAT ACT      95
TCA GAG TCT CCC ATT AGA ATA TCA GTA TCC ACA GAA GGA GCA AAT ACT
                                                  A

Ser Ser Ser
TCT TCA T                                                          102
TCT TCA T
```

FIG. 7
SEGMENT C: (SEQ ID NOS:19-22)

```
   Thr Ser Thr Ser Thr Ala Gly Thr Ser His Leu Val Lys Cys Ala
CC ACA TCC ACA TCT ACA GCT GGG ACA AGC CAT CTT GTC AAG TGT GCA      47
CT ACA TCT ACA TCC ACC ACT GGG ACA AGC CAT CTT GTA AAA TGT GCG
                           T

Glu Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys Phe Met Val
GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGC GAG TGC TTC ATG GTG      95
GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGG GAG TGC TTC ATG GTG

Lys Asp Leu Ser Asn Pro Ser Arg Tyr Leu Cys
AAA GAC CTT TCA AAT CCC TCA AGA TAC TTG TGC                         128
AAA GAC CTT TCA AAC CCC TCG AGA TAC TTG TGC
```

FIG. 8
SEGMENT C/D: (SEQ ID NOS:23-26)

```
Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn Val Pro
AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT GTG CCC      48
||| ||| ||| ||| ||| ||| ||| ||| ||  ||| ||| ||| ||| ||| ||| |||
AAG TGC CAA CCT GGA TTC ACT GGA GCA AGA TGT ACT GAG AAT GTG CCC

Met Lys Val Gln Thr Gln Glu
ATG AAA GTC CAA ACC CAA GAA                                        69
||| ||| ||| ||| | | ||| |||
ATG AAA GTC CAA AAC CAA GAA
              N
```

FIG. 9
SEGMENT C/D': (SEQ ID NOS:27-29)

```
Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met
AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG      48
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG

Ala Ser Phe Tyr
GCC AGC TTC TAC                                                    60
||| ||| ||| |||
GCC AGC TTC TAC
```

FIG. 10
SEGMENT D: (SEQ ID NOS:30-32)

```
Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro Glu  *
AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA TAG                     36
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA TAG
```

FIG. 11
SEGMENT D': (SEQ ID NOS:33-34)

```
Lys His Leu Gly Ile Glu Phe Met Glu
AAG CAT CTT GGG ATT GAA TTT ATG GAG                    27
```

FIG. 12A
SEGMENT H: (SEQ ID NOS:35-38)

```
Lys Ala Glu Glu Leu Tyr Gln Lys Arg Val Leu Thr Ile Thr Gly Ile
AAA GCG GAG GAG CTC TAC CAG AAG AGA GTG CTC ACC ATT ACC GGC ATT        48
||| ||| ||| ||| ||  ||| ||| ||| ||| ||| ||  ||| ||  ||| ||| ||
AAA GCG GAG GAG CTG TAC CAG AAG AGA GTG CTG ACC ATA ACC GGC ATC

Cys Ile Ala Leu Leu Val Val Gly Ile Met Cys Val Val Val Tyr Cys
TGC ATC GCG CTG CTC GTG GTT GGC ATC ATG TGT GTG GTG GTC TAC TGC        96
||| ||| ||  ||  ||  ||| ||  ||| ||| ||| ||| ||| ||| | | ||| |||
TGC ATC GCC CTC CTT GTG GTC GGC ATC ATG TGT GTG GTG GCC TAC TGC
                                          A

Lys Thr Lys Lys Gln Arg Lys Lys Leu His Asp Arg Leu Arg Gln Ser
AAA ACC AAG AAA CAA CGG AAA AAG CTT CAT GAC CGG CTT CGG CAG AGC       144
||| ||| ||| ||| ||  ||| ||| ||| ||  ||| ||| ||  ||| ||| ||| |||
AAA ACC AAG AAA CAG CGG AAA AAG CTG CAT GAC CGT CTT CGG CAG AGC

Leu Arg Ser Glu Arg Asn Thr Met Met Asn Val Ala Asn Gly Pro His
CTT CGG TCT GAA AGA AAC ACC ATG ATG AAC GTA GCC AAC GGG CCC CAC       192
||| ||| ||| ||| ||  ||| |   ||| ||| |||  |  ||| ||  ||| ||  |||
CTT CGG TCT GAA CGA AAC AAT ATG ATG AAC ATT GCC AAT GGG CCT CAC
                    N               I

His Pro Asn Pro Pro Pro Glu Asn Val Gln Leu Val Asn Gln Tyr Val
CAC CCC AAT CCG CCC CCC GAG AAC GTG CAG CTG GTG AAT CAA TAC GTA       240
||  ||  ||  ||  ||| ||| ||| ||  ||  ||| ||| ||| ||| ||| ||| |||
CAT CCT AAC CCA CCC CCC GAG AAT GTC CAG CTG GTG AAT CAA TAC GTA

Ser Lys Asn Val Ile Ser Ser Glu His Ile Val Glu Arg Glu Ala Glu
TCT AAA AAT GTC ATC TCT AGC GAG CAT ATT GTT GAG AGA GAG GCG GAG       288
||| ||| ||  ||| ||| ||  ||  ||| ||| ||| ||| ||| ||| ||  ||  |||
TCT AAA AAC GTC ATC TCC AGT GAG CAT ATT GTT GAG AGA GAA GCA GAG

Ser Ser Phe Ser Thr Ser His Tyr Thr Ser Thr Ala His His Ser Thr
AGC TCT TTT TCC ACC AGT CAC TAC ACT TCG ACA GCT CAT CAT TCC ACT       336
|   ||  ||| ||| ||| ||| ||| ||  ||| ||  ||| ||  ||| ||  ||| |||
ACA TCC TTT TCC ACC AGT CAC TAT ACT TCC ACA GCC CAT CAC TCC ACT
T
```

FIG. 12B

```
Thr Val Thr Gln Thr Pro Ser His Ser Trp Ser Asn Gly His Thr Glu
ACT GTC ACT CAG ACT CCC AGT CAC AGC TGG AGC AAT GGA CAC ACT GAA      384
ACT GTC ACC CAG ACT CCT AGC CAC AGC TGG AGC AAC GGA CAC ACT GAA

Ser Ile Ile Ser Glu Ser His Ser Val Ile Val Met Ser Ser Val Glu
AGC ATC ATT TCG GAA AGC CAC TCT GTC ATC GTG ATG TCA TCC GTA GAA      432
AGC ATC CTT TCC GAA AGC CAC TCT GTA ATC GTG ATG TCA TCC GTA GAA
        L

Asn Ser Arg His Ser Ser Pro Thr Gly Gly Pro Arg Gly Arg Leu Asn
AAC AGT AGG CAC AGC AGC CCG ACT GGG GGC CCG AGA GGA CGT CTC AAT      480
AAC AGT AGG CAC AGC AGC CCA ACT GGG GGC CCA AGA GGA CGT CTT AAT

Gly Leu Gly Gly Pro Arg Glu Cys Asn Ser Phe Leu Arg His Ala Arg
GGC TTG GGA GGC CCT CGT GAA TGT AAC AGC TTC CTC AGG CAT GCC AGA      528
GGC ACA GGA GGC CCT CGT GAA TGT AAC AGC TTC CTC AGG CAT GCC AGA
    T

Glu Thr Pro Asp Ser Tyr Arg Asp Ser Pro His Ser Glu Arg
GAA ACC CCT GAC TCC TAC CGA GAC TCT CCT CAT AGT GAA AG              569
GAA ACC CCT GAT TCC TAC CGA GAC TCT CCT CAT AGT GAA AG
```

FIG. 13
SEGMENT K: (SEQ ID NOS:39-40)

```
A CAT AAC CTT ATA GCT GAG CTA AGG AGA AAC AAG GCC CAC AGA TCC       46
  His Asn Leu Ile Ala Glu Leu Arg Arg Asn Lys Ala His Arg Ser
    1               5                  10                  15

AAA TGC ATG CAG ATC CAG CTT TCC GCA ACT CAT CTT AGA GCT TCT TCC      94
Lys Cys Met Gln Ile Gln Leu Ser Ala Thr His Leu Arg Ala Ser Ser
                20                  25                  30

ATT CCC CAT TGG GCT TCA TTC TCT AAG ACC CCT TGG CCT TTA GGA AG      141
Ile Pro His Trp Ala Ser Phe Ser Lys Thr Pro Trp Pro Leu Gly Arg
            35                  40                  45
```

FIG. 14A
SEGMENT L: (SEQ ID NOS:41-44)

```
  Tyr Val Ser Ala Met Thr Thr Pro Ala Arg Met Ser Pro Val Asp
G TAT GTA TCA GCA ATG ACC ACC CCG GCT CGT ATG TCA CCT GTA GAT      46
| ||| ||  ||| ||  ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
G TAT GTG TCA GCC ATG ACC ACC CCG GCT CGT ATG TCA CCT GTA GAT

Phe His Thr Pro Ser Ser Pro Lys Ser Pro Pro Ser Glu Met Ser Pro
TTC CAC ACG CCA AGC TCC CCC AAG TCA CCC CCT TCG GAA ATG TCC CCG     94
||| ||| ||| ||| ||| ||| ||| ||  ||  ||| ||| ||| ||| ||| ||| ||
TTC CAC ACG CCA AGC TCC CCC AAA TCG CCC CCT TCG GAA ATG TCT CCA

Pro Val Ser Ser Thr Thr Val Ser Met Pro Ser Met Ala Val Ser Pro
CCC GTG TCC AGC ACG ACG GTC TCC ATG CCC TCC ATG GCG GTC AGT CCC    142
||| ||| ||| ||| | | ||| ||  ||| ||| ||  ||| ||| ||| ||| ||  |||
CCC GTG TCC AGC ATG ACG GTG TCC ATG CCT TCC ATG GCG GTC AGC CCC
                M

Phe Val Glu Glu Glu Arg Pro Leu Leu Leu Val Thr Pro Pro Arg Leu
TTC GTG GAA GAG GAG AGA CCC CTG CTC CTT GTG ACG CCA CCA CGG CTG    190
|||  ||  ||| ||  ||| ||| ||  ||  ||  ||  ||| ||  ||| ||| ||  |||
TTC ATG GAA GAA GAG AGA CCT CTA CTT CTC GTG ACA CCA CCA AGG CTG
    N

Arg Glu Lys  -  Tyr Asp His His Ala Gln Gln Phe Asn Ser Phe His
CGG GAG AAG ... TAT GAC CAC CAC GCC CAG CAA TTC AAC TCG TTC CAC    238
||| ||| |||     | |  ||| ||  |||  |  |||  ||  |||  |  ||  ||| |||
CGG GAG AAG AAG TTT GAC CAT CAC CCT CAG CAG TTC AGC TCC TTC CAC
            K   F               P

Cys Asn Pro Ala His Glu Ser Asn Ser Leu Pro Pro Ser Pro Leu Arg
TGC AAC CCC GCG CAT GAG AGC AAC AGC CTG CCC CCC AGC CCC TTG AGG    286
  |  ||| ||| ||| ||| ||  ||  ||| ||| ||  ||  |  ||| ||| ||| |||
CAC AAC CCC GCG CAT GAC AGT AAC AGC CTC CCT GCT AGC CCC TTG AGG
N                   D                   A

Ile Val Glu Asp Glu Glu Tyr Glu Thr Thr Gln Glu Tyr Glu Pro Ala
ATA GTG GAG GAT GAG GAA TAT GAA ACG ACC CAG GAG TAC GAA CCA GCT    334
||| ||| ||| ||| ||| ||  ||| ||| ||| ||| ||  ||| ||| ||  ||| ||
ATA GTG GAG GAT GAG GAG TAT GAA ACG ACC CAA GAG TAC GAG CCA GCC

Gln Glu Pro Val Lys Lys Leu Thr Asn Ser Ser Arg Arg Ala Lys Arg
CAA GAG CCG GTT AAG AAA CTC ACC AAC AGC AGC CGG CGG GCC AAA AGA    382
||| ||| ||  ||| ||| ||| ||| ||  |||   . ||| ||| ||| ||| ||| |||
CAA GAG CCT GTT AAG AAA CTC GCC AA. ..T AGC CGG CGG GCC AAA AGA
                          A
```

FIG. 14B

```
Thr Lys Pro Asn Gly His Ile Ala His Arg Leu Glu Met Asp Asn Asn
ACC AAG CCC AAT GGT CAC ATT GCC CAC AGG TTG GAA ATG GAC AAC AAC      430
||| ||| ||| ||| ||  ||| ||| ||  ||  ||  ||| ||| ||  ||| | | |||
ACC AAG CCC AAT GGC CAC ATT GCT AAC AGA TTG GAA GTG GAC AGC AAC
                               N               V       S

Thr Gly Ala Asp Ser Ser Asn Ser Glu Ser Glu Thr Glu Asp Glu Arg
ACA GGC GCT GAC AGC AGT AAC TCA GAG AGC GAA ACA GAG GAT GAA AGA      478
|||  ||  |   |  ||| ||| ||| ||| ||| ||  ||| ||| ||  ||| ||| |||
ACA AGC TCC CAG AGC AGT AAC TCA GAG AGT GAA ACA GAA GAT GAA AGA
    S   S   Q

Val Gly Glu Asp Thr Pro Phe Leu Ala Ile Gln Asn Pro Leu Ala Ala
GTA GGA GAA GAT ACG CCT TTC CTG GCC ATA CAG AAC CCC CTG GCA GCC      526
|||  ||  ||| ||| ||| ||| ||| ||| |  | ||| ||| ||| ||| ||| ||| |||
GTA GGT GAA GAT ACG CCT TTC CTG GGC ATA CAG AAC CCC CTG GCA GCC
                               G

Ser Leu Glu Ala Ala Pro Ala Phe Arg Leu Val Asp Ser Arg Thr Asn
AGT CTC GAG GCG GCC CCT GCC TTC CGC CTG GTC GAC AGC AGG ACT AAC      574
|||  ||  ||| ||  |  ||| ||| ||| ||| ||| |  ||| ||| ||| ||| |||
AGT CTT GAG GCA ACA CCT GCC TTC CGC CTG GCT GAC AGC AGG ACT AAC
                T                       A

Pro Thr Gly Gly Phe Ser Pro Gln Glu Glu Leu Gln Ala Arg Leu Ser
CCA ACA GGC GGC TTC TCT CCG CAG GAA GAA TTG CAG GCC AGG CTC TCC      622
|||  ||  |||  ||  ||| ||  |  ||| ||| ||| |  ||| ||| ||| ||  ||
CCA GCA GGC CGC TTC TCG ACA CAG GAA GAA ATC CAG GCC AGG CTG TCT
    A       R           T               I

Gly Val Ile Ala Asn Gln Asp Pro Ile Ala Val  *
GGT GTA ATC GCT AAC CAA GAC CCT ATC GCT GTC TAA AAC CGA AAT ACA      670
 ||  ||| ||  ||| ||| ||| ||| ||| ||  ||| ||  ||| ||| | | ||| | |
AGT GTA ATT GCT AAC CAA GAC CCT ATT GCT GTA TAA AAC CTA AAT AAA
S

CCC ATA GAT TCA CCT GTA AAA CTT TAT TTT ATA TAA TAA AGT ATT CCA      718
| | ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
CAC ATA GAT TCA CCT GTA AAA CTT TAT TTT ATA TAA TAA AGT ATT CCA

CCT TAA ATT AAA CAA                                                 733
||| ||| ||| ||| |||
CCT TAA ATT AAA CAA
```

FIG. 15
SEGMENT F: (SEQ ID NOS:45-48)

```
AGTTTCCCCC CCCAACTTGT CGGAACTCTG GGCTCGCGCG CAGGGCAGGA GCGGAGCGGC      60

GGCGGCTGCC CAGGCGATGC GAGCGCGGGC CGGACGGTAA TCGCCTCTCC CTCCTCGGGC     120

TGCGAGCGCG CCGGACCGAG GCAGCGACAG GAGCGGACCG CGGCGGGAAC CGAGGACTCC     180

CCAGCGGCGC GCCAGCAGGA GCCACCCCGC GAGNCGTGCG ACCGGGACGG AGCGCCCGCC     240

AGTCCCAGGT GGCCCGGACC GCACGTTGCG TCCCCGCGCT CCCCGCCGGC GACAGGAGAC     300

GCTCCCCCCC ACGCCGCGCG CGCCTCGGCC CGGTCGCTGG CCCGCCTCCA CTCCGGGGAC     360
                |||| | |||||| ||  ||| |||| | | | |  ||  |||  |||||
                CGCGAG CGCCTCAGCG CGGCCGCTCG CTCTC..CCC CTCGAGGGAC

AAACTTTTCC CGAAGCCGAT CCCAGCCCTC GGACCCAAAC TTGTCGCGCG TCGCCTTCGC     420
|||||||||| | || ||||| || |||||| |||||| ||           |||||| |||
AAACTTTTCC CAAACCCGAT CCGAGCCCTT GGACCAAA.. .........C TCGCCTGCGC

                                                Met Ser Glu Arg Arg
CGGGAGCCGT CCGCGCAGAG CGTGCACTTC TCGGGCGAG ATG TCG GAG CGC AGA         474
|| |||||||| ||||| |||| || | | || || ||||||| ||| ||  ||| ||| | |
CGAGAGCCGT CCGCGTAGAG CGCTC.CGTC TCCGGCGAG ATG TCC GAG CGC AAA
                                                          K

Glu Gly Lys Gly Lys Gly Lys Gly Gly Lys Lys Asp Arg Gly Ser Gly
GAA GGC AAA GGC AAG GGG AAG GGC GGC AAG AAG GAC CGA GGC TCC GGG       522
||| ||| | | ||| ||  ||| ||| |||     ||| ||| ||  ||| ||| ||| ||
GAA GGC AGA GGC AAA GGG AAG GGC AAG AAG AAG GAG CGA GGC TCC GGC
        R                       K           E

Lys Lys Pro Val Pro Ala Ala Gly Gly Pro Ser Pro Ala
AAG AAG CCC GTG CCC GCG GCT GGC GGC CCG AGC CCA G                     559
||| ||| ||  | |  || ||| ||  |||  ||  | | ||| ||| |
AAG AAG CCG GAG TCC GCG GCG GGC AGC CAG AGC CCA G
          E   S
```

FIG. 16A
(SEQ ID NOS:49-50)

```
G AAG TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA      49
  Lys Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu
   1               5                  10                  15

TAT ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC      97
Tyr Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala
            20                  25                  30

AAC ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG     145
Asn Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly
        35                  40                  45

ACA AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG     193
Thr Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val
    50                  55                  60

AAT GGA GGC GAC TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA     241
Asn Gly Gly Asp Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg
65                  70                  75                  80

TAC TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG     289
Tyr Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu
                85                  90                  95

AAT GTG CCC ATG AAA GTC CAA ACC CAA GAA AAA GCG GAG GAG CTC TAC     337
Asn Val Pro Met Lys Val Gln Thr Gln Glu Lys Ala Glu Glu Leu Tyr
            100                 105                 110

CAG AAG AGA GTG CTC ACC ATT ACC GGC ATT TGC ATC GCG CTG CTC GTG     385
Gln Lys Arg Val Leu Thr Ile Thr Gly Ile Cys Ile Ala Leu Leu Val
        115                 120                 125

GTT GGC ATC ATG TGT GTG GTG GTC TAC TGC AAA ACC AAG AAA CAA CGG     433
Val Gly Ile Met Cys Val Val Val Tyr Cys Lys Thr Lys Lys Gln Arg
    130                 135                 140

AAA AAG CTT CAT GAC CGG CTT CGG CAG AGC CTT CGG TCT GAA AGA AAC     481
Lys Lys Leu His Asp Arg Leu Arg Gln Ser Leu Arg Ser Glu Arg Asn
145                 150                 155                 160

ACC ATG ATG AAC GTA GCC AAC GGG CCC CAC CAC CCC AAT CCG CCC CCC     529
Thr Met Met Asn Val Ala Asn Gly Pro His His Pro Asn Pro Pro Pro
                165                 170                 175

GAG AAC GTG CAG CTG GTG AAT CAA TAC GTA TCT AAA AAT GTC ATC TCT     577
Glu Asn Val Gln Leu Val Asn Gln Tyr Val Ser Lys Asn Val Ile Ser
            180                 185                 190
```

FIG. 16B

```
AGC GAG CAT ATT GTT GAG AGA GAG GCG GAG AGC TCT TTT TCC ACC AGT          625
Ser Glu His Ile Val Glu Arg Glu Ala Glu Ser Ser Phe Ser Thr Ser
        195                 200                 205

CAC TAC ACT TCG ACA GCT CAT CAT TCC ACT ACT GTC ACT CAG ACT CCC          673
His Tyr Thr Ser Thr Ala His His Ser Thr Thr Val Thr Gln Thr Pro
    210                 215                 220

AGT CAC AGC TGG AGC AAT GGA CAC ACT GAA AGC ATC ATT TCG GAA AGC          721
Ser His Ser Trp Ser Asn Gly His Thr Glu Ser Ile Ile Ser Glu Ser
225                 230                 235                 240

CAC TCT GTC ATC GTG ATG TCA TCC GTA GAA AAC AGT AGG CAC AGC AGC          769
His Ser Val Ile Val Met Ser Ser Val Glu Asn Ser Arg His Ser Ser
                245                 250                 255

CCG ACT GGG GGC CCG AGA GGA CGT CTC AAT GGC TTG GGA GGC CCT CGT          817
Pro Thr Gly Gly Pro Arg Gly Arg Leu Asn Gly Leu Gly Gly Pro Arg
            260                 265                 270

GAA TGT AAC AGC TTC CTC AGG CAT GCC AGA GAA ACC CCT GAC TCC TAC          865
Glu Cys Asn Ser Phe Leu Arg His Ala Arg Glu Thr Pro Asp Ser Tyr
        275                 280                 285

CGA GAC TCT CCT CAT AGT GAA AGA CAT AAC CTT ATA GCT GAG CTA AGG          913
Arg Asp Ser Pro His Ser Glu Arg His Asn Leu Ile Ala Glu Leu Arg
    290                 295                 300

AGA AAC AAG GCC CAC AGA TCC AAA TGC ATG CAG ATC CAG CTT TCC GCA          961
Arg Asn Lys Ala His Arg Ser Lys Cys Met Gln Ile Gln Leu Ser Ala
305                 310                 315                 320

ACT CAT CTT AGA GCT TCT TCC ATT CCC CAT TGG GCT TCA TTC TCT AAG         1009
Thr His Leu Arg Ala Ser Ser Ile Pro His Trp Ala Ser Phe Ser Lys
                325                 330                 335

ACC CCT TGG CCT TTA GGA AGG TAT GTA TCA GCA ATG ACC ACC CCG GCT         1057
Thr Pro Trp Pro Leu Gly Arg Tyr Val Ser Ala Met Thr Thr Pro Ala
            340                 345                 350

CGT ATG TCA CCT GTA GAT TTC CAC ACG CCA AGC TCC CCC AAG TCA CCC         1105
Arg Met Ser Pro Val Asp Phe His Thr Pro Ser Ser Pro Lys Ser Pro
        355                 360                 365

CCT TCG GAA ATG TCC CCG CCC GTG TCC AGC ACG ACG GTC TCC ATG CCC         1153
Pro Ser Glu Met Ser Pro Pro Val Ser Ser Thr Thr Val Ser Met Pro
    370                 375                 380
```

FIG. 16C

```
TCC ATG GCG GTC AGT CCC TTC GTG GAA GAG GAG AGA CCC CTG CTC CTT          1201
Ser Met Ala Val Ser Pro Phe Val Glu Glu Glu Arg Pro Leu Leu Leu
385                 390                 395                 400

GTG ACG CCA CCA CGG CTG CGG GAG AAG TAT GAC CAC CAC GCC CAG CAA          1249
Val Thr Pro Pro Arg Leu Arg Glu Lys Tyr Asp His His Ala Gln Gln
                405                 410                 415

TTC AAC TCG TTC CAC TGC AAC CCC GCG CAT GAG AGC AAC AGC CTG CCC          1297
Phe Asn Ser Phe His Cys Asn Pro Ala His Glu Ser Asn Ser Leu Pro
            420                 425                 430

CCC AGC CCC TTG AGG ATA GTG GAG GAT GAG GAA TAT GAA ACG ACC CAG          1345
Pro Ser Pro Leu Arg Ile Val Glu Asp Glu Glu Tyr Glu Thr Thr Gln
        435                 440                 445

GAG TAC GAA CCA GCT CAA GAG CCG GTT AAG AAA CTC ACC AAC AGC AGC          1393
Glu Tyr Glu Pro Ala Gln Glu Pro Val Lys Lys Leu Thr Asn Ser Ser
    450                 455                 460

CGG CGG GCC AAA AGA ACC AAG CCC AAT GGT CAC ATT GCC CAC AGG TTG          1441
Arg Arg Ala Lys Arg Thr Lys Pro Asn Gly His Ile Ala His Arg Leu
465                 470                 475                 480

GAA ATG GAC AAC AAC ACA GGC GCT GAC AGC AGT AAC TCA GAG AGC GAA          1489
Glu Met Asp Asn Asn Thr Gly Ala Asp Ser Ser Asn Ser Glu Ser Glu
                485                 490                 495

ACA GAG GAT GAA AGA GTA GGA GAA GAT ACG CCT TTC CTG GCC ATA CAG          1537
Thr Glu Asp Glu Arg Val Gly Glu Asp Thr Pro Phe Leu Ala Ile Gln
            500                 505                 510

AAC CCC CTG GCA GCC AGT CTC GAG GCG GCC CCT GCC TTC CGC CTG GTC          1585
Asn Pro Leu Ala Ala Ser Leu Glu Ala Ala Pro Ala Phe Arg Leu Val
        515                 520                 525

GAC AGC AGG ACT AAC CCA ACA GGC GGC TTC TCT CCG CAG GAA GAA TTG          1633
Asp Ser Arg Thr Asn Pro Thr Gly Gly Phe Ser Pro Gln Glu Glu Leu
    530                 535                 540

CAG GCC AGG CTC TCC GGT GTA ATC GCT AAC CAA GAC CCT ATC GCT GTC          1681
Gln Ala Arg Leu Ser Gly Val Ile Ala Asn Gln Asp Pro Ile Ala Val
545                 550                 555                 560

TAAAACCGAA ATACACCCAT AGATTCACCT GTAAAACTTT ATTTTATATA ATAAAGTATT        1741

CCACCTTAAA TTAAACAAAA AAA                                               1764
```

FIG. 17A
(SEQ ID NOS:51-52)

```
CAT CAA GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG          48
His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu
1               5                   10                  15

CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC GCC TTC CCC TCC TGC          96
Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser Cys
            20                  25                  30

GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAG         144
Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro Glu
        35                  40                  45

GCC AAC AGC AGC GGC GGG CCC GGC CGC CTT CCG AGC CTC CTT CCC CCC         192
Ala Asn Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro Pro
    50                  55                  60

TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT GTG         240
Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala Val
65                  70                  75                  80

CAA CGG TGC GCC TTG CCT CCC CGC TTG AAA GAG ATG AAG AGT CAG GAG         288
Gln Arg Cys Ala Leu Pro Pro Arg Leu Lys Glu Met Lys Ser Gln Glu
                85                  90                  95

TCT GTG GCA GGT TCC AAA CTA GTG CTT CGG TGC GAG ACC AGT TCT GAA         336
Ser Val Ala Gly Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu
            100                 105                 110

TAC TCC TCT CTC AAG TTC AAG TGG TTC AAG AAT GGG AGT GAA TTA AGC         384
Tyr Ser Ser Leu Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser
        115                 120                 125

CGA AAG AAC AAA CCA GAA AAC ATC AAG ATA CAG AAA AGG CCG GGG AAG         432
Arg Lys Asn Lys Pro Glu Asn Ile Lys Ile Gln Lys Arg Pro Gly Lys
    130                 135                 140

TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT         480
Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr
145                 150                 155                 160

ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC         528
Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn
                165                 170                 175
```

FIG. 17B

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ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA          576
Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly Thr
            180                 185                 190

AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT          624
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
        195                 200                 205

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC          672
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
    210                 215                 220

TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT          720
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn
225                 230                 235                 240

GTG CCC ATG AAA GTC CAA ACC CAA GAA AAG TGC CCA AAT GAG TTT ACT          768
Val Pro Met Lys Val Gln Thr Gln Glu Lys Cys Pro Asn Glu Phe Thr
                245                 250                 255

GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC TAC AGT ACG TCC          816
Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe Tyr Ser Thr Ser
            260                 265                 270

ACT CCC TTT CTG TCT CTG CCT GAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG         870
Thr Pro Phe Leu Ser Leu Pro Glu
        275                 280

TTGCCGCATC TCCCCTCAGA TTCCNCCTAG AGCTAGATGC GTTTTACCAG GTCTAACATT        930

GACTGCCTCT GCCTGTCGCA TGAGAACATT AACACAAGCG ATTGTATGAC TTCCTCTGTC        990

CGTGACTAGT GGGCTCTGAG CTACTCGTAG GTGCGTAAGG CTCCAGTGTT TCTGAAATTG       1050

ATCTTGAATT ACTGTGATAC GACATGATAG TCCCTCTCAC CCAGTGCAAT GACAATAAAG       1110

GCCTTGAAAA GTCAAAAAAA AAAAAAAAAA                                        1140
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FIG. 18A
(SEQ ID NOS:53-54)

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AGTTTCCCCC CCCAACTTGT CGGAACTCTG GGCTCGCGCG CAGGGCAGGA GCGGAGCGGC      60

GGCGGCTGCC CAGGCGATGC GAGCGCGGGC CGGACGGTAA TCGCCTCTCC CTCCTCGGGC     120

TGCGAGCGCG CCGGACCGAG GCAGCGACAG GAGCGGACCG CGGCGGGAAC CGAGGACTCC     180

CCAGCGGCGC GCCAGCAGGA GCCACCCCGC GAGCGTGCGA CCGGGACGGA GCGCCCGCCA     240

GTCCCAGGTG GCCCGGACCG CACGTTGCGT CCCCGCGCTC CCCGCCGGCG ACAGGAGACG     300

CTCCCCCCCA CGCCGCGCGC GCCTCGGCCC GGTCGCTGGC CCGCCTCCAC TCCGGGGACA     360

AACTTTTCCC GAAGCCGATC CCAGCCCTCG GACCCAAACT TGTCGCGCGT CGCCTTCGCC     420

GGGAGCCGTC CGCGCAGAGC GTGCACTTCT CGGGCGAG ATG TCG GAG CGC AGA          473
                                          Met Ser Glu Arg Arg
                                            1               5

GAA GGC AAA GGC AAG GGG AAG GGC GGC AAG AAG GAC CGA GGC TCC GGG        521
Glu Gly Lys Gly Lys Gly Lys Gly Gly Lys Lys Asp Arg Gly Ser Gly
                  10                  15                  20

AAG AAG CCC GTG CCC GCG GCT GGC GGC CCG AGC CCA GCC TTG CCT CCC        569
Lys Lys Pro Val Pro Ala Ala Gly Gly Pro Ser Pro Ala Leu Pro Pro
            25                  30                  35

CGC TTG AAA GAG ATG AAG ATG CAG GAG TCT GTG GCA GGT TCC AAA CTA        617
Arg Leu Lys Glu Met Lys Ser Gln Glu Ser Val Ala Gly Ser Lys Leu
        40                  45                  50

GTG CTT CGG TGC GAG ACC AGT TCT GAA TAC TCC TCT CTC AAG TTC AAG        665
Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser Ser Leu Lys Phe Lys
    55                  60                  65

TGG TTC AAG AAT GGG AGT GAA TTA AGC CGA AAG AAC AAA CCA CAA AAC        713
Trp Phe Lys Asn Gly Ser Glu Leu Ser Arg Lys Asn Lys Pro Gln Asn
70                  75                  80                  85

ATC AAG ATA CAG AAA AGG CCG GGG AAG TCA GAA CTT CGC ATT AGC AAA        761
Ile Lys Ile Gln Lys Arg Pro Gly Lys Ser Glu Leu Arg Ile Ser Lys
                90                  95                 100

GCG TCA CTG GCT GAT TCT GGA GAA TAT ATG TGC AAA GTG ATC AGC AAA        809
Ala Ser Leu Ala Asp Ser Gly Glu Tyr Met Cys Lys Val Ile Ser Lys
            105                 110                 115
```

FIG. 18B

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CTA GGA AAT GAC AGT GCC TCT GCC AAC ATC ACC ATT GTG GAG TCA AAC        857
Leu Gly Asn Asp Ser Ala Ser Ala Asn Ile Thr Ile Val Glu Ser Asn
        120                 125                 130

GAG ATC ACC ACT GGC ATG CCA GCC TCA ACT GAG ACA GCG TAT GTG TCT        905
Glu Ile Thr Thr Gly Met Pro Ala Ser Thr Glu Thr Ala Tyr Val Ser
    135                 140                 145

TCA GAG TCT CCC ATT AGA ATA TCA GTA TCA ACA GAA GGA ACA AAT ACT        953
Ser Glu Ser Pro Ile Arg Ile Ser Val Ser Thr Glu Gly Thr Asn Thr
150                 155                 160                 165

TCT TCA TCC ACA TCC ACA TCT ACA GCT GGG ACA AGC CAT CTT GTC AAG       1001
Ser Ser Ser Thr Ser Thr Ser Thr Ala Gly Thr Ser His Leu Val Lys
                170                 175                 180

TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGC GAG TGC TTC       1049
Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys Phe
            185                 190                 195

ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC TTG TGC AAG TGC CCA       1097
Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr Leu Cys Lys Cys Pro
        200                 205                 210

AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC       1145
Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe
    215                 220                 225

TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA TAGGCGCATG            1191
Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro Glu
230                 235                 240

CTCAGTCGGT GCCGCTTTCT TGTTGCCGCA TCTCCCCTCA GATTCAACCT AGAGCTAGAT    1251

GCGTTTTACC AGGTCTAACA TTGACTGCCT CTGCCTGTCG CATGAGAACA TTAACACAAG    1311

CGATTGTATG ACTTCCTCTG TCCGTGACTA GTGGGCTCTG AGCTACTCGT AGGTGCGTAA    1371

GGCTCCAGTG TTTCTGAAAT TGATCTTGAA TTACTGTGAT ACGACATGAT AGTCCCTCTC    1431

ACCCAGTGCA ATGACAATAA AGGCCTTGAA AAGTCTCACT TTTATTGAGA AAATAAAAAT    1491

CGTTCCACGG GACAGTCCCT CTTCTTTATA AAATGACCCT ATCCTTGAAA AGGAGGTGTG    1551

TTAAGTTGTA ACCAGTACAC ACTTGAAATG ATGGTAAGTT CGCTTCGGTT CAGAATGTGT    1611

TCTTTCTGAC AAATAAACAG AATAAAAAAA AAAAAAAAAA A                        1652
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/21349

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/70, 38/00, 38/02, 38/18
US CL :514/2, 12, 44, 903, 907

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 12, 44, 903, 907

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,530,109 A (GOODEARL et al.) 25 June 1996, column 3, line 3 to column 6, line 53 and column 11, line 1 to column 12, line 59.	1-34

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 DECEMBER 1998	Date of mailing of the international search report 05FEB1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer STEPHEN GUCKER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/21349

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 35-36
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, SCISEARCH, EMBASE, BIOSIS, CAPLUS, WPIDS, BIOTECHDS, CONFSCI, LIFESCI
neuregulin#, glia#, heregulin#, GGF#, ischemia#, dementia#, Parkinson#, Huntington#, Alzheimer#, infarct#, amyotrophic, Down#, Korsakoff#, heart#, cardiac, spinal

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, LR, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHODS OF TREATING DISORDERS OF THE EYE

(57) Abstract

The present invention relates to methods for the prophylaxis or treatment of retinal cells by the administration of a therapeutically effective amount of a neuregulin polypeptide.

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METHODS OF TREATING DISORDERS OF THE EYE

GOVERNMENT SUPPORT

This invention was made with the support of a federal grant from the U.S. Government (Grant No. 5ROINS28308-06). The Government has certain rights in the invention.

FIELD OF THE INVENTION

This invention relates to methods of affecting retinal cell function.

BACKGROUND OF THE INVENTION

The invention relates to prophylactic or affirmative treatment of diseases and disorders of retina and associated tissues of the eye by administering polypeptides found in vertebrate species, which polypeptides are growth, differentiation and survival factors for several cell types. Normal function of retinal cells including survival, proliferation, differentiation, and maintenance is dependent upon the controlled expression of a variety of peptide growth factors. Some of these factors can be produced by neuronal cells and by other cells of the retina, which provide a signal to regulate retinal cell function.

Anatomy and Function of the Retina

The retina is that component of the visual system which senses light and transmits impulses via the optic nerve to the visual cortex where the signals are deciphered and interpreted as images. The retina is comprised of a series of layers and cell types as illustrated in Figure 1.

The basic function of the retina is to transduce the visual image into a pattern of electrical potential changes that can be processed by the visual centers in the brain. The changes in electrical potentials in the retinal cells are then relayed to the brain. The structure of the retina reflects these functions (Figure 1). The cells of the retina are arrayed in three layers: (1) the outer nuclear layer, which contains the photoreceptor cells; (2) the inner nuclear layer, which contains the cell nuclei of most of the retinal interneurons and glia; and (3) the ganglion cell layer, which contains the cell bodies of the cells that relay the visual information to the brain via the optic nerve. In addition to these nuclear layers, there are three other distinct layers in the retina. The outermost layer is composed of the outer

segments of the photoreceptor cells; this is where the actual process of light-to-electrical signal transduction take place. The outer plexiform layer lies between the outer and inner nuclear layers. It is made up of synapses between the terminals of the photoreceptors and the dendrites of the retinal interneurons of the inner nuclear layer. The inner plexiform layer lies between the inner nuclear layer and the ganglion cell layer. This layer is where the interneurons of the inner nuclear layer synapse with the retinal ganglion cell dendrites.

The retina is composed of five classes of neurons, and two classes of supporting cells (*Principles of Neural Science*, 3rd ed., Ed. by E.R. Kandel, J. H. Schwartz, and T. M. Jessell, Elsevier, New York, NY 1991). Of the neuronal types, the receptor cells are the cells that transduce light into electrical signals. Receptor cells are of two subtypes: cones - which mediate form and color perception in daylight, and rods - which mediate form perception in dim light. Ganglion cells of the retina project axons into the brain via the optic nerve and are the output cells of the retina. The remaining neuronal types are interneurons that modulate retinal output: bipolar cells connect receptor cells to ganglion cells; horizontal cells mediate lateral interactions between receptors and bipolar cells; and amacrine cells mediate lateral interactions between bipolar cells and ganglion cells. The supporting cell types are the glial cells of the retina, Müller cells, and the pigment epithelium cells. The latter cell type plays an important role in the maintenance of receptor cells.

The basic flow of information through the retina is as follows (Refer to Figure 1): (1) light passes through the cells of the retina and is absorbed by the outer segments of the photoreceptor cells; (2) the photons are transduced into potential changes in the photoreceptor cells; (3) this change in potential is relayed to one type of retinal interneuron in the inner nuclear layer, the bipolar cell, via synapses in the outer plexiform layer; (4) the bipolar cells relay the electrical potential changes to the ganglion cells through their synapses in the inner plexiform layer; and (5) the ganglion cells convert the potential changes into action potentials that are sent along the optic nerve to the brain. This process results in a pattern of action potentials in the optic nerves that reflects the pattern of light and dark in the visual world. Some initial processing of the visual information takes place in the retina before it is relayed to the other visual areas in the brain.

Proper development and maintenance of the retina is necessary for sustaining normal vision. Degeneration of components of the retina can lead to partial or total blindness.

Peptide Growth Factors

The development and physiology of multicellular organisms requires multiple modes of intercellular communication. Such communication may be systemic, as in the case of hormones delivered via the bloodstream, or can be highly localized. In the latter case two modes are commonly recognized: synaptic signaling from neurons, and paracrine signaling from adjacent or nearby cells (*Molecular Biology of the Cell*, Alberts et al., 2nd ed. Garland Publishing, New York, NY 1989). A function of such signaling is to coordinate cell survival, proliferation, differentiation, and/or metabolic activity. The molecules that serve as transmitted signals vary in their chemical composition; one group of molecules are proteins, the peptide growth factors. Peptide growth factors act upon cells by binding to cell surface receptors. These receptors are coupled to intracellular signal transduction pathways that give rise to the above described activities when activated by growth factor binding. The genesis and differentiation of the varied retinal cell types and the generation of distinct layers in the retina from progenitor cells of the optic cup are the result of developmental events that are mediated by intercellular communication involving peptide growth factors.

Peptide Growth Factors in the retina

The roles of growth factors in the development and maintenance of the retina have been studied in cell culture, by molecular analysis of the expressed growth factors and their receptors, and in animal models of disease or injury.

As an example of *in vitro* studies, explants and partially-dissociated chick retinal pigmented epithelium (RPE) can trans-differentiate into neural retina in the presence of bFGF (Coulombre and Coulombre, *Dev. Biol.* 12:79, 1965). Proliferation of dissociated RPE cells is stimulated by αFGF, βFGF, EGF, PDGF, IGF, and insulin; and it is inhibited by TGFß (Sternfeld et al., *Curr. Eye Res.* 8: 1029, 1989; Leschey et al., *Invest. Ophthalmol. Vis. Sci.* 31: 839, 1990; Song and Lui, *J. Cell Physiol.* 143:196, 1990). Cultured RPE cells are induced by cytokines to release nitric oxide, which is cytotoxic--and the induction can be blocked by FGF (Goureau et al., *Biochem. Biophys Res. Comm.* 186:854, 1992; op. cit., 198: 120, 1994). Further, retinal explants from the *rd* mouse are rescued from cell death by combined treatment with NGF and bFGF (Caffe et al., *Curr. Eye Res.* 12:719, 1993).

The presence of growth factor receptors in retinal cells has been demonstrated by a variety of molecular analytical techniques, including immunostaining, *in situ* hybridization and tissue binding using radio-labeled ligands. Cells in the RPE express FGF receptors

(Malecaze et al., *J. Cell Physiol.* 154: 1105, 1993). Ganglion cells and Müller cells express receptors for BDNF, CNTF, FGF, trkA and trkB (Jelsma et al., *J. Neurobiol.* 24:1207, 1993; Takahashi et al., *Neurosci. Lett.* 151:174, 1993; Carmignoto et al., *Exp. Neurol.* 111:302 191; reviewed in Steinberg, *Curr. Opin. Neurobiol.* 4:515, 1994). Müller cells also express PDGF receptors (Mudhar et al., *Development* 118: 539, 1993). Receptors for IGF are detected on photoreceptor cells (Waldbillig et al., *Exp. Eye Res.* 47:587 1988; Ocrant et al., *Exp. Eye Res.* 52:581, 1991), and depending on the species and developmental stage that are analyzed receptors for bFDF have been localized on several cell types, including retinal ganglion cells (Sternfeld et al., *Curr. Eye Res.* 8:1029, 1992; Schweigerer et al., *Biochem Biophys. Res. Comm.* 143:934, 1987).

Studies on retinal ganglion cell survival *in vivo* in animal models of optic nerve axotomy and retinal ischemia have demonstrated effects due to FGF (Sievers et al., *Neurosci. Lett.* 76:157, 1987), NGF (Carmignoto et al., *J. Neurosci.* 9:1263, 1989), CNTF (Mey and Thanos, *Brain Res.* 602:304, 1993), BDNF (Mansour-Robaey et al., *PNAS USA* 91:1632, 1994; Mey and Thanos, *Brain Res.* 602:304, 1993), NT4/5 (Cohen et al., *J. Neurobiol.* 25:953, 1994) and bFGF (Ferguson et al., *J. Neurosci.* 10:2176, 1990). Some undesirable retinal complications, including macrophage proliferation, inflammation, disorganization of retinal structure and angiogenesis are associated with treatment of the retina with several of the above factors.

Neuregulins

A recently described family of growth factors, the neuregulins (reviewed by Mudge, *Curr. Biol.* 3:361, 1993; Peles and Yarden, *Bioessays* 15:815, 1993), are synthesized by neurons (Marchionni et al. *Nature* 362:313, 1993) and by mesenchymal cells from several parenchymal organs (Meyer and Birchmeier, *PNAS* 91:1064, 1994). The neuregulins and related factors that bind $p185^{erbB2}$ have been purified, cloned and expressed (Benveniste et al. *PNAS*, 82:3930, 1985; Kimura et al., *Nature* 348:257, 1990; Davis and Stroobant, *J. Cell Biol.* 110:1353, 1990; Wen et al., *Cell* 69:559, 1992; Yarden and Ullrich, *Ann. Rev. Biochem.* 57:443, 1988; Dobashi et al., *Proc. Natl. Acad. Sci.* 88:8582, 1991: Lupu et al., *Proc. Natl. Acad. Sci.* 89:2287, 1992; Wen et al., *Mol. Cell. Biol.* 14:1909, 1994). Recombinant neuregulins have been shown to be mitogenic for peripheral glia (Marchionni et al., *Nature* 362:313, 1993) and have been shown to influence the formation of the neuromuscular junction (Falls et al., *Cell* 72:801, 1993; Jo et al., *Nature* 373: 158, 1995; Chu et al., *Cell* 14: 329, 1995).

The neuregulin gene consists of at least thirteen exons. The neuregulin transcripts are alternatively spliced and these encode many distinct peptide growth factors, which are referred to as the neuregulins (Marchionni et al., *Nature* 362:313, 1993). DNA sequence comparisons revealed that neu differentiation factor (NDF) (Wen et al., *Cell* 69:559, 1992) and heregulins (Holmes et al., *Science* 256:1205, 1992), which were purified as ligands of the $p185^{erbB2}$ (also known as neu or HER2) receptor tyrosine kinase, also are splicing variants of the neuregulin gene. The acetylcholine receptor inducing activity (ARIA) also is a product of the neuregulin gene (Falls et al., *Cell* 72:801, 1993). Common structural features of the neuregulins are the presence of a single immunoglobulin-like (Ig) fold and a single epidermal growth factor-like (EGF) domain.

The sites of neuregulin gene expression have been characterized by use of nucleic acid probes to analyze RNA samples by a variety of methods, such as Northern blotting, RNase protection, or *in situ* hybridization. Transcripts have been detected in the nervous system and in a variety of other tissues (Holmes et al., *Science* 256:1205, 1992 Wen et al., *Cell* 69:559, 1992; Meyer and Birchmeier, *PNAS* 91:1064, 1994). Sites of gene expression have been localized in the brain and spinal chord and in other tissues. (Orr-Urteger et al., *PNAS* 90:1867, 1993; Falls et al., *Cell* 72:801, 1993; Marchionni et al., *Nature* 362:313, 1993; Meyer and Birchmeier, *PNAS* 91:1064, 1994 ; Chen et al., *J. Comp. Neurol.* 349; 389, 1994; Corfas et al., *Neuron* 14:103, 1995). Specifically in the retinal neurepithelium, expression of neuregulin transcripts has been detected at embryonic day 18 in rat (Meyer and Birchmeier, *PNAS* 91:1064, 1994).

Although a large number of neuregulins may be produced by alternative splicing, they can be broadly sorted into the putative membrane-bound and the soluble isoforms. The former contains a putative trans-membrane domain and may be presented at the cell surface. Membrane-anchored peptide growth factors may mediate cell-cell interactions through cell-adhesion or juxtacrine mechanisms (reviewed by Massagué and Pandiella, *Ann. Rev. Biochem.* 62:515, 1993). Alternatively, the putative membrane-bound isoforms may be cleaved from the cell surface and function as soluble proteins (Wen et al., *Cell* 69:559, 1992; Falls et al., *Cell* 72:801, 1993). The soluble neuregulin isoforms contain sequence corresponding to the extracellular domains of the putative membrane-bound isoforms, but terminate before the transmembrane domain. These neuregulin isoforms may be secreted, and hence could affect cells at a distance; or they may be present in the cytoplasm, but could be released upon cellular injury. In the latter case, neuregulins may function as injury factors, as has been postulated for the ciliary neurotrophic factor (Stöckli et al., *Nature*

342:920, 1989). Any one of these modes of action of the neuregulins may occur in the retina.

Cellular targets of peptide growth factors are those which bear receptors for the factor(s) and those that are shown to respond in a bioassay either *in vitro* or *in vivo*. Based on studies demonstrating phosphorylation on tyrosine residues or cross-linking experiments, neuregulins are candidate ligands for the receptor tyrosine kinases $p185^{erbB2}$ (or HER-2 in human), $p185^{erbB3}$ (HER-3 in human), $p185^{erbB4}$ (or HER-4 in human) or related members of the EGFR gene family. Collectively, these receptors can be referred to as erbB receptors. Though the precise ligand-receptor relationship of each neuregulin protein with each member of the EGFR family is yet to be clarified, several lines of evidence suggest that binding of ligands is mediated by either erbB3 and erbB4 , but signaling occurs through either erbB2, erbB4 and heterodimers of the various subunits (e.g., Carraway and Cantley, *Cell* 78:5, 1994). These receptors are known to be present on Schwann cells and muscle cells (Jo et al., *Nature* 373: 158, 1995), and other neuregulin targets, such as cell lines derived from various tumor tissues, such as breast and gastric epithelia. Sites of expression of the HER-4 gene have been localized by *in situ* hybridization to several regions of the brain, including: hippocampus, dentate gyrus, neo cortex, medial habenula, reticular nucleus of the thalamus, and the amygdala (Lai and Lemke, *Neuron* 6:691, 1991). The distribution of the HER-4 receptor has not been studied by methods that allow detection of the protein or the activated receptor tyrosine kinase *in vivo* or in cultures of primary cells. The expression pattern of erbB2, erbB3 and erbB4 in the retina has not been described.

Neuregulins have been shown to have a variety of biological activities depending on the cell type being studied. Several neuregulins, including native bovine GGFI, II and III and recombinant human GGF2 (rhGGF2) are mitogenic for Schwann cells (Marchionni et al., *Nature* 362:313, 1993), as is heregulin B1 (Levi et al, *J Neurosci.* 15:1329, 1995). On human muscle culture, rhGGF2 has a potent trophic effect on myotubes (Sklar et al., U.S. Pat. Applic. # 08/059, 022). The differentiation response to rhGGF2 also includes induction of acetylcholine receptors in cultured myotubes (Jo et al., *Nature* 373: 158, 1995). This activity is associated with other forms of neuregulin, including ARIA (Falls et al., *Cell* 72:801, 1993) and heregulin B1 (Chu et al., *Neuron* 14:329, 1995), as well as with rhGGF2. Further, ARIA has been shown to induce synthesis of voltage-gated sodium channels in chick skeletal muscle (Corfas and Fischbach, *J. Neurosci.* 13:2118, 1993). Glial growth factor (GGF), and more specifically rhGGF2, can restrict neural crest stem cells to differentiate into glial cells *in vitro* (Shah et al., *Cell* 77:349, 1994). Activities of neuregulin

on retinal cells have not been described. In summary, there are examples of neuregulin proteins affecting proliferation, survival and differentiation of target cells.

Pharmaceutical need for treating disorders of the eye

A variety of retinal diseases and related disorders are known that produce impaired vision and in some cases progress to total blindness. These disorders of the eye include, but are not necessarily limited to: various retinopathies, such as hypertensive retinopathy, diabetic retinopathy and occlusive retinopathy; also injuries and disorders resulting in retinal degeneration, such as retinal tearing and detachment and inherited diseases, such as retinitis pigmentosa; also age-related macular degeneration; diseases of the optic nerve; glaucoma and retinal ischemia.

Diabetic Retinopathy is the leading cause of blindness in patients 25-74 years. It is responsible for 12,000-24,000 new cases of blindness per year in the United States. Of the 6 million diabetics in the US 50% show detectable retinopathy after 7 years of diabetes. Age-related macular degeneration (ARMD) is estimated to be present in over 9% of the population 52 years and older and in 33% of the population 75 years and older. Glaucoma is associated with chronically high intraocular pressure and approximately 2 million people in the US are currently being treated. In the US approximately 100,000 people are blinded each year by glaucoma.

There is precedent for the use of growth factors that have been shown to be active on retinal cultures in the treatment of retinal degenerative diseases. FGF supports the survival of photoreceptor cells in culture and has been injected into the extracellular space surrounding the rods and cones or into the vitreous body to rescue the photoreceptors in rats which have degeneration as a result of light damage or because of an inherited disease (LaVail et al, *PNAS* 89: 11249, 1992, Faktorovich et al *J. NeuroSci* 12: 3554, 1992). Similarly TGFß2 has been used for the treatment of Macular holes in humans. The TGFß used was derived from bovine sources and was administered by directly infusing the factor into the area of the macular hole (Glaser et al., *Opthalmol.* 99: 1162, 1992).

Currently, there are limited options for therapy for the promotion of retinal cell function, including survival, proliferation, differentiation, growth and changes in gene activity and metabolic activity. Such a therapy would be useful for treatment of a variety of eye disorders resulting in loss of sight.

SUMMARY OF THE INVENTION

In general, the present invention provides methods for promoting the function of retinal cells using neuregulins. A novel aspect of the invention involves the use of neuregulins as growth factors to promote survival of retinal cells. Treating of the retinal cells to provide these effects may be achieved by contacting retinal cells with a polypeptide described herein. The treatments may be provided to slow or halt net cell loss or to increase the amount or quality of retinal tissue present in the vertebrate.

Neuregulins are a family of protein factors heretofore described as glial growth factors, acetylcholine receptor inducing activity (ARIA), heregulins, neu differentiation factor, which are encoded by one gene. A variety of messenger RNA splicing variants (and their resultant proteins) are derived from this gene and many of these products show binding to and activation of erbB2 (neu) and closely related receptors erbB3 and erbB4. The invention provides methods for using all of the known products of the neuregulin gene, as well as, other not yet discovered splicing variants of the neuregulin gene. Thus, the above factors, regulatory compounds that induce synthesis of these factors, and small molecules which mimic the effect of these factors by binding to the receptors on retinal tissues or by stimulating through other means the second messenger systems activated by the ligand-receptor complex are all extremely useful as prophylactic and affirmative therapies for retinal tissue diseases and related disorders of the eye.

The survival of retinal cells as used herein refers to the prevention of loss of retinal cells by necrosis or apoptosis or the prevention of other mechanisms of retinal loss. Survival as used herein indicates a decrease in the rate of cell death of at least 10%, more preferably by at least 50%, and most preferably by at least 100% relative to an untreated control. The rate of survival may be measured by counting cells stainable with a dye specific for dead cells (such as propidium iodide) in culture.

Methods for treatment of diseases or disorders using the polypeptides or other compounds described herein are also part of the invention. Examples of retinal tissue disorders that may be treated include eye diseases and disorders resulting from sensorineural pathologies, such as loss of sight, which may also be treated using the methods of the invention. These disorders of the eye include, but are not necessarily limited to: various retinopathies, such as hypertensive retinopathy, diabetic retinopathy and occlusive retinopathy; also injuries and disorders resulting in retinal degeneration, such as retinal

tearing and detachment and inherited diseases, such as retinitis pigmentosa; also age-related macular degeneration: diseases of the optic nerve; glaucoma and retinal ischemia.

The methods of the invention make use of the fact that the various neuregulin proteins are encoded by the same gene. A variety of messenger RNA splicing variants (and their resultant proteins) are derived from this gene and many of these products show binding to $p185^{erbB2}$ (or related receptors erbB3 and erbB4) and activation of the same. Products of this gene are used to show retinal cell survival activity (see Example 2, below). This invention provides a use for all of the known products of the neuregulin gene (described herein and in the references listed above), which have the stated activities as promoting retinal cell function. Most preferably, recombinant human GGF2 (rhGGF2) is used in these methods.

The invention also relates to the use of other, not yet naturally isolated, splicing variants of the neuregulin gene. Figure 12 shows the known patterns of splicing. These patterns are derived from polymerase chain reaction experiments (on reverse transcribed RNA), analysis of cDNA clones (as presented within) and from analysis of published sequences encoding neuregulins (Peles et al., *Cell* 69:205, 1992;Wen et al., *Cell* 69:559, 1992; Wen et al., *Mol. Cell Biol.* 14:1909, 1994) These patterns, as well as additional patterns disclosed herein, represent probable splicing variants which exist. The splicing variants are fully described in Goodearl et al., USSN 08/036,555, filed March 24, 1993, incorporated herein by reference.

Advantages of the present invention include the development of new therapeutic approaches to injury or diseases of the eye, more specifically degenerative diseases of the retina, based on the promotion of retinal cell function through the use of neuregulins. Loss of retinal cells is a common feature of degenerative eye diseases, and there are no available treatments, including growth factors , that prevent the death of retinal ganglion cells. The factor can be formulated for intraocular injection and administered to patients that suffer from degenerative disorders , which lead to loss of sight. Thus, this approach to therapy can halt or slow the progressive loss of sight, which ensues in various eye diseases.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the series of layers and cell types which form the retina: (1) corresponds to the ganglion cell layer; (2) corresponds to the inner plexiform layer; (3) corresponds to the inner nuclear layer; (4) corresponds to the outer plexiform layer; (5) corresponds to the outer nuclear layer.

Figure 2 is immunostaining showing that neuregulin protein is expressed in the retinal ganglion cell layer during embryonic retinal development. Arrows point to labeling in the developing ganglion cell layer.

Figure 3 is *in situ* hybridization showing that neuregulin mRNA is expressed in cells of the retinal ganglion cell layer during embryonic development. Arrows point to the labeling in the ganglion cell layer, showing that the distribution is similar to the neuregulin immunoreactivity shown in Figure 2.

Figure 4 is immunostaining showing that neuregulin protein is present in the inner and outer plexiform layers of the adult retina.

Figure 5 is immunostaining showing that TUJ1 immunoreactivity is expressed in the newborn rat retina and shows that retinal ganglion cells are the primary cell class that expresses this antigen at this stage of development. The retinal ganglion cell layer is marked with large arrows, while the labeled amacrine cells are marked with the small arrows and the labeled horizontal cells are marked with the arrowheads.

Figure 6 is an immunostained culture of rat retinal neurons, which were grown for two days in the presence of rhGGF2 (neuregulin) on collagen gels showing extended long processes labeled with the TUJ1 antibody.

Figure 7 is an immunostained culture of rat retinal cells showing that neuregulin (rhGGF2) causes a significant increase in TUJ1 immunoreactive in embryonic day 18 rat retinal cells after two days of culture.

Figure 8 is an immunostained culture of rat retinal cells showing that the neuregulin (rhGGF2)- induced cell survival is age dependent: neuregulin (rhGGF2) does not cause a significant increase in TUJ1 immunoreactive embryonic day 15 rat retinal cells after two days of culture.

Figure 9 is a bar graph of the results of three separate experiments with embryonic day 18 rat retinal cells.

Figure 10 is a bar graph of the experimental results showing the effects of GGF on retinal cell survival.

Figure 11A is a listing of the coding strand DNA sequence and deduced amino aid sequence of the cDNA obtained from the splicing pattern of GGFBPP1 shown in Figure 12. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA);

Figure 11B is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from splicing pattern of GGF2BPP2. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA);

Figure 11C is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from splicing pattern of GGF2BPP3. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA).

Figure 12 shows products of the neuregulin gene.

Figure 13 is a listing of the DNA sequences and predicted peptide sequences of the coding segments of GGF. Line 1 is a listing of the predicted amino acid sequences of bovine GGF, line 2 is a listing of the nucleotide sequences of bovine GGF, line 3 is a listing of the nucleotide sequences of human GGF (heregulin) (nucleotide base matches are indicated with a vertical line) and line 4 is a listing of the predicted amino acid sequences of human GGF/heregulin where it differs from the predicted bovine sequence. Coding segments E, A' and K represent only the bovine sequences. Coding segment D' represents only the human (heregulin) sequence.

Figure 14 is the predicted GGF2 amino acid sequence and nucleotide sequence of BPP5. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

Figure 15 is the predicted amino acid sequence and nucleotide sequence of GGF2BPP2. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

Figure 16 is the predicted amino acid sequence and nucleotide sequence of GGF2BPP4. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

Figure 17 is a list of splicing variants derived from the sequences shown in Figure 13.

Figure 18 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL1.

Figure 19 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL2.

Figure 20 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL3.

Figure 21 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL4.

Figure 22 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL5.

Figure 23 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL6.

Figure 24 is the predicted amino acid sequence (middle) and nucleic sequence (top) of GGF2HBS5. The bottom (intermittent) sequence represents peptide sequences derived from GGF-II preparations.

Figure 25 is the sequences of GGFHBS5, GGFHB1 and GGFBPP5 polypeptides.

Figure 26 is the amino acid sequence of cDNA encoding mature hGGF2.

Figure 27 depicts a stretch of the putative bovine GGF-II gene sequence from the recombinant bovine genomic phage GGF2BG1. The figure is the coding strand of the DNA sequence and the deduced amino acid sequence in the third reading frame.

DETAILED DESCRIPTION OF THE INVENTION

It is intended that all references cited shall be incorporated herein by reference.

General

The invention pertains to methods of promoting function of retinal cells. The function is affected by the administration of a neuregulin to a vertebrate where the neuregulin interacts with a retinal cell to promote one or more aspects of retinal cell function, including proliferation, differentiation, growth, survival, changes in the pattern of gene expression and secretion, and metabolic change of the retinal cell.

Definition of key terms

The term administration as used herein refers to the act of delivering a substance, including but not limited to the following routes: parenteral, intravenous, subcutaneous, intramuscular, intraorbital, ophthalmic, intravitreal, subretinal, intraperitoneal, topical, intranasal, aerosol or oral.

The term affecting as used herein refers to the induction of a quantitative change in the response of a target cell, as a result of an interaction with a neuregulin.

The term amacrine cell as used herein refers to local interneurons in the inner plexiform layer of the retina that mediate interactions between bipolar and ganglion cells.

The term bipolar cell as used herein refers to the interneurons of the retina that connect the photoreceptor cells with the retinal ganglion cells.

The term differentiation as used herein refers to a morphological and/or chemical change that results in the generation of a different cell type or state of specialization. The differentiation of cells as used herein refers to the induction of a cellular developmental program which specifies one or more components of a cell type. The therapeutic usefulness of differentiation can be seen, in increases in quantity of any component of a cell type in diseased tissue by at least 10% or more, more preferably by 50% or more, and most preferably by more than 100% relative to the equivalent tissue in a similarly treated control animal.

The term disorder as used herein refers to a disturbance of function and/or structure of a living organism, resulting from an external source, a genetic predisposition, a physical or chemical trauma, or a combination of the above, including but not limited to any mammalian disease.

The term erbB receptor as used herein refers to erbB2, erbB3 and erbB4 (also HER-2, HER-3 and HER-4 of human) existing as monomeric, homodimeric and heterodimeric (e.g., erbB2/erbB3) cell surface receptor tyrosine kinases that bind and/or are activated by one or more neuregulins.

The term function as used herein refers to any activity or response of a cell. These include but are not limited to proliferation, differentiation, growth, survival, changes in the pattern of gene expression and secretion, and metabolic changes.

The term horizontal cell used herein refers to local interneurons in the outer plexiform layer of the retina that mediate interactions between bipolar and receptors cells.

The term mammal as used herein describes a member of the Class Mammalia (Subphylum Vertebrata).

The term mitosis as used herein refers to the division of a cell where each daughter nucleus receives identical complements of the numbers of chromosomes characteristic of the somatic cells of the species. Mitosis as used herein refers to any cell division which results in the production of new cells in the patient. More specifically, a useful therapeutic is defined *in vitro* as an increase in mitotic index relative to untreated cells of 50%, more preferably 100%, and most preferably 300%, when the cells are exposed to labeling agent for a time equivalent to two doubling times. The mitotic index is the fraction of cells in the culture which have labeled nuclei when grown in the presence of a tracer which only incorporates during S phase (i.e., BrdU) and the doubling time is defined as the average time required for the number of cells in the culture to increase by a factor of two.

The term neuregulin as used herein refers to the glial growth factors, the heregulins, neu differentiation factor, acetylcholine receptor inducing activity, and erbB2, 3 and 4 binding proteins. A more complete definition of neuregulins can be found in the specification herein and in the following materials: U.S. Patent No. 5,237,056; U.S. Patent Application SN 08/249,322; WO 92/20798; EPO 0 505 148 A1; Marchionni, et al., *Nature* 362:313, 1993; Benveniste, et al., *PNAS* 82:3930, 1985; Kimura, et al., *Nature* 348:257,

1990; Davis and Stroobant, *J. Cell. Biol.* 110:1353, 1990; Wen, et al., *Cell* 69:559, 1992; Yarden and Ullrich, *Ann. Rev. Biochem.* 57:443, 1988; Holmes, et al., *Science* 256:1205, 1992; Dobashi, et al., *Proc. Natl. Acad. Sci.* 88:8582, 1991; Lupu, et al., *Proc. Natl. Acad. Sci.* 89:2287, 1992; Peles and Yarden, *BioEssays* 15:815, 1993, Mudge, *Current Biology* 3:361, 1993, all hereby incorporated by reference.

The term neurological disorder as described herein refers to a disorder of the nervous system.

The term photoreceptor cell as used herein refers to two retinal cell types, rods and cones, that are the cells that transduce light into an electrical signal.

The term retinal cell as used herein refers to any of the cell types that comprise the retina, such as retinal ganglion cells, amacrine cells, horizontal cells, bipolar cells, and photoreceptor cells including rods and cones, Müller glial cells and retinal pigmented epithelium.

The term retinal ganglion cell as used herein refers to neurons of the retina that project axons via the optic nerve to the lateral geniculate nucleus and the superior colliculus.

The term survival as used herein refers to any process where a cell avoids death. The term survival as used herein also refers to the prevention of cell loss as evidenced by necrosis or apoptosis or the prevention of other mechanisms of cell loss. Survival as used herein indicates a decrease in the rate of cell death by at least 10%, more preferably by at least 50%, and most preferably by at least 100% relative to an untreated control. The rate of survival may be measured by counting cells stainable with a dye specific for dead cells (such as propidium iodide) in culture.

The term therapeutically effective amount as used herein refers to that amount which will produce a desirable result upon administration and which will vary depending upon a number of issues, including the dosage to be administered, and the route of administration.

The term treating as used herein may refer to a procedure (e.g. medical procedure) designed to exert a beneficial effect on a disorder. Treating as used herein means any administration of a substance described herein for the purpose of increasing retinal cell function. Most preferably, the treating is for the purpose of reducing or diminishing the symptoms or progression of a disease or disorder of retinal cells. Treating as used herein

also means the administration of a substance to increase or alter the cells in healthy individuals. The treating may be brought about by the contacting of the cells which are sensitive or responsive to the neuregulins described herein with an effective amount of the neuregulin.

The term TUJ1 as used herein refers to an antibody that recognizes a neural-specific form of ß-tubulin, which is expressed in the longitudinal cells, amacrine cells and ganglion cells of the retina.

The term vertebrate as used herein refers to an animal that is a member of the Subphylum Vertebrata (Phylum Chordata).

Neuregulins

A novel aspect of the present invention relates to the ability of neuregulins to affect retinal cell function. Neuregulins are the products of a gene which produce a number of variably-sized, differentially-spliced RNA transcripts that give rise to a series of proteins. These proteins are of different lengths and contain some common peptide sequences and some unique peptide sequences. The conclusion that these factors are encoded by a single gene is supported by the differentially-spliced RNA sequences which are recoverable from bovine posterior pituitary, human spinal chord and human breast cancer cells (MDA-MB-231). Further support for this conclusion derives from the size range of proteins which act as ligands for the erbB receptors (see below).

Further evidence to support the fact a single gene encodes the various neuregulins derives from nucleotide sequence comparisons. Holmes et al., (*Science* 256:1205, 1992) demonstrate the purification of a 45-kilodalton human protein (Heregulin-a) which specifically interacts with the receptor protein p185erbB2. Peles et al., (*Cell* 69:559, 1992) describe a complementary DNA isolated from rat cells encoding a protein call "neu differentiation factor" (NDF). The translation product of the NDF cDNA has p185erbB2 binding activity. Several other groups have reported the purification of proteins of various molecular weights with erbB receptor binding activity. These groups include the following: Lupu et al., *Proc. Natl. Acad. Sci. USA* 89:2287, 1992; Yarden and Peles, *Biochemistry* 30:3543, 1991; Lupu et al., *Science* 249:1552, 1990; Dobashi et al., *Biochem. Biophys. Res. Comm.* 179:1536, 1991; and Huang et al., *J. Biol. Chem.* 257:11508, 1992.

We have found that proteins that bind p185erbB2 and related receptors (i.e., p185erbB3 and p185erbB4) affect retinal cell survival (Example 2). Further, the presence of immunologically-detectable neuregulin protein (Example 1) in retinal ganglion cells *in vivo* indicates that neuregulin has a role in retinal cell survival *in vivo*.

These neuregulins may be identified using the protocols described herein and in Holmes et al., *Science* 256: 1205, 1992; Peles et al., *Cell* 69:205, 1992; Wen et al., *Cell* 69:559, 1992; Lupu et al., *Proc. Natl. Acad. Sci. USA* 89:2287, 1992; Yarden and Peles, *Biochemistry* 30:3543, 1991; Lupu et al., *Science* 249:1552, 1990; Dobashi et al., *Biochem. Biophys. Res. Comm.* 179:1536, 1991; Huang et al., *J. Biol. Chem.* 257:11508-11512, 1992; Marchionni et al., *Nature* 362:313, 1993; and in U.S. Patent Application Serial No. 07/931,041, filed August 17, 1992, all of which are incorporated herein by reference.

Specifically, the invention provides for use of polypeptides of a specified formula, and DNA sequences encoding those polypeptides. The polypeptides have the formula

WYBAZCX

wherein WYBAZCX is composed of the amino acid sequences shown in Figure 13; wherein W comprises the polypeptide segment F, or is absent: wherein Y comprises the polypeptide segment E, or is absent; wherein Z comprises the polypeptide segment G or is absent: and wherein X comprises the polypeptide segments C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' D' HKL; provided that, either

a) at least one of F, Y, B, A, Z, C, or X is of bovine origin; or

b) Y comprises the polypeptide segment E; or

c) X comprises the polypeptide segments C/D HKL, C/D D, C/D' HKL, C/D C/D' HKL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, C/D C/D' D' HKL, C/D'H, C/D C/D'H, or C/D C/D' HL.

In addition, the invention includes the use of the DNA sequence comprising coding segments $^{5'}$FBA$^{3'}$ as well as the with corresponding polypeptide segments having the amino acid sequences shown in Figure 13;

the DNA sequence comprising the coding segments $^{5'}$FBA'$^{3'}$ as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 13;

the DNA sequence comprising the coding segments $^{5'}$FEBA$^{3'}$ as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 13;

the DNA sequence comprising the coding segments $^{5'}$FEBA'$^{3'}$ as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 13;

the DNA sequence comprising the polypeptide coding segments of the GGF2HBS5 cDNA clone (ATCC Deposit No. 75298, deposited September 2, 1992), also known as GGF-II.

The invention further includes the use of peptides of the formula FBA, FEBA, FBA' FEBA' and DNA sequences encoding these peptides wherein the polypeptide segments correspond to amino acid sequences shown in Figure 13. The polypeptide purified GGF-II polypeptide is also included as part of the invention.

Also included in this invention is the mature GGF peptide and the DNA encoding said peptide, exclusive of the N-terminal signal sequence, which is also useful for treatment of conditions involving abnormalities in retinal cell function.

Furthermore, the invention includes a method of retinal cell function by the application to a vertebrate of a

- 30 kD polypeptide factor isolated from the MDA - MB 231 human breast cell line; or

- 35 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line to the glial cell; or

-75 kD polypeptide factor isolated from the SKBR-3 human breast cell line; or

-44 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line, or

-25 kD polypeptide factor isolated from activated mouse peritoneal macrophages; or

-45 kD polypeptide factor isolated from the MDA - MB 231 human breast cell; or

-7 to 14 kD polypeptide factor isolated from the ATL-2 human T-cell line to the glial cell; or

-25 kD polypeptide factor isolated from the bovine kidney cell; or

-42 kD polypeptide factor (ARIA) isolated from brains.

The invention further includes a method for the use of the EGFL1, EGFL2, EGFL3, EGFL4, EGFL5, and EGFL6 polypeptides, Figures 18 to 23 and respectively, for the methods of affecting retinal cell function *in vivo* and *in vitro*.

Also included in the invention is the administration of the GGF-II polypeptide whose sequence is shown in Figure 24 for affecting retinal cell function.

Thus, the invention further embraces a polypeptide factor capable of affecting retinal cell function and including an amino acid sequence encoded by:

(a) a DNA sequence shown in Figure 11;

(b) a DNA sequence shown in Figure 27;

(c) the DNA sequence represented by nucleotides 281-557 of the sequences shown in Figure 11; or

(d) a DNA sequence hybridizable to any one of the DNA sequences according to (a), (b) or (c).

The invention further includes sequences which have greater than 60%, preferably 80%, sequence identity of homology to the sequences indicated above.

While the present invention is not limited to a particular set of hybridization conditions, the following protocol gives general guidance which may, if desired, be followed:

DNA probes may be labeled to high specific activity (approximately 10^8 to 10^9 ^{32}P dpm/μg) by nick-translation or by PCR reactions according to Schowalter and Sommer (*Anal. Biochem.* 177:90, 1989) and purified by desalting on G-150 Sephadex columns. Probes may be denatured (10 minutes in boiling water followed by immersion into ice water), then added to hybridization solutions of 80% buffer B (2g polyvinylpyrolidine, 2g Ficoll-400, 2g bovine serum albumin, 50ml 1 M Tris HCL (pH 7.5), 58g NaCl, 1g sodium pyrophosphate, 10g sodium dodecyl sulfate, 950 ml H_2O) containing 10% dextran sulfate at 10^6 dpm ^{32}P per ml and incubated overnight (approximately 16 hours) at 60° C. The filters may then be washed at 60° C first in buffer B for 15 minutes followed by three 20-minute washes in 2X SSC, 0.1% SDS then one for 20 minutes in 1XSSC, 0.1% SDS.

In other respects, the invention provides:

(a) a basic polypeptide factor which has, if obtained from bovine pituitary material, an observed molecular weight, whether in reducing conditions or not, of from about 30 kD to about 36 kD on SDS-polyacrylamide gel electrophoresis using the following molecular weight standards:

Lysozyme (hen egg white)	14,400
Soybean trypsin inhibitor	21,500
Carbonic anhydrase (bovine)	31,000
Ovalbumin (hen egg white)	45,000
Bovine serum albumin	66,200
Phosphorylase B (rabbit muscle)	97,400;

which factor has glial cell mitogenic activity including stimulating the division of rat Schwann cells in the presence of fetal calf plasma, and when isolated using reversed-phase HPLC retains at least 50% of said activity after 10 weeks incubation in 0.1 % trifluoroacetic acid at 4° C; and

(b) a basic polypeptide factor which has, if obtained from bovine pituitary material, an observed molecular weight, under non-reducing conditions, or from about 55 kD to about 63 kD on SDS-polyacrylamide gel electrophoresis using the following molecular weight standards:

Lysozyme (hen egg white)	14,400
Soybean trypsin inhibitor	21,500
Carbonic anhydrase (bovine)	31,000
Ovalbumin (hen egg white)	45,000
Bovine serum albumin	66,200
Phosphorylase B (rabbit muscle)	97,400;

which factor the human equivalent of which is encoded by DNA clone GGF2HBS5 described herein and is capable of affecting retinal cell function.

For convenience of description only, the lower molecular weight and higher molecular weight factors of this invention are referred to hereafter as "GGF-I" and "GGF-II", respectively. The "GGF2" designation is used for all clones isolated with peptide sequence data derived from GGF-II protein (i.e., GGF2HBS5, GGF2BPP3).

It will be appreciated that the molecular weight range limits quoted are not exact, but are subject to slight variations depending upon the source of the particular polypeptide factor. A variation of, say, about 10% would not, for example, be impossible for material from another source.

Another important aspect of the invention is a DNA sequence encoding a polypeptide capable of affecting retinal cell function and comprising:

(a) a DNA sequence shown Figure 11;

(b) a DNA sequence shown in Figure 27;

(c) the DNA sequence represented by nucleotides 281-557 of the sequence shown in Figure 11; or

(d) a DNA sequence hybridizable to any one of the DNA sequences according to (a), (b) or (c).

Thus other important aspects of the invention are:

(a) A series of human and bovine polypeptide factors capable of affecting retinal cell function. These peptide sequences are shown in Figures 13, 14, 15 and 16 respectively.

(b) A series of polypeptide factors capable of affecting retinal cell function and purified and characterized according to the procedures outlined by Lupu et al., *Science* 249:1552, 1990; Lupu et al., *Proc. Natl. Acad. Sci USA* 89: 2287, 1992; Holmes et al., *Science* 256:1205, 1992; Peles et al., *Cell* 69:205, 1992; Yarden and Peles, *Biochemistry* 30:3543, 1991; Dobashi et al., *Proc. Natl. Acad. Sci.* 88: 8582, 1991; Davis et al., *Biochem.*

Biophys. Res. Commun. 179:1536, 1991; Beaumont et al., Patent Application PCT/US91/03443 (1990); Greene et al., Patent Application PCT/US91/02331 (1990); Usdin and Fischbach, *J. Cell. Biol.* 103:493, 1986; Falls et al., *Cold Spring Harbor Symp. Quant. Biol.* 55:397, 1990; Harris et al., *Proc. Natl. Acad. Sci. USA* 88:7664, 1991; and Falls et al., *Cell* 72:801, 1993.

(c) A polypeptide factor (GGFBPP5) is capable of affecting retinal cell function. The amino acid sequence is shown in Figure 14, and is encoded by the bovine DNA sequence shown in Figure 14.

The novel human peptide sequences described above and presented Figures 13, 14, 15, and 16, respectively, represent a series of splicing variants which can be isolated as full length complementary DNAs (cDNAs) from natural sources (cDNA libraries prepared from the appropriate tissues) or can be assembled as DNA constructs with individual exons (e.g., derived as separate exons) by someone skilled in the art.

Other compounds, in particular peptides, which bind specifically to erbB receptors can also be used according to the invention as effectors of retinal cell function. A candidate compound can be routinely screened for erbB receptor binding, and, if it binds, can then be screened for affecting retinal cell function, more specifically, retinal cell survival, using the methods described herein.

The invention includes any modifications or equivalents of the above polypeptide factors which do not exhibit a significantly reduced activity. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting activity are included. By way of illustration, in EP-A 109748 mutations of native proteins are disclosed in which the possibility of unwanted disulfide bonding is avoided by replacing any cysteine in the native sequence which is not necessary for biological activity with a neutral amino acid. The statements of effect and use contained herein are therefore to be construed accordingly, with such uses and effects employing modified or equivalent factors being part of the invention.

The new sequences of the invention open up the benefits of recombinant technology. The invention thus also includes the following aspects:

(a) DNA constructs comprising DNA sequences as defined above in operable reading frame position within vectors (positioned relative to control sequences so as to permit expression of the sequences) in chosen host cells after transformation thereof by the constructs (preferably the control sequence includes regulatable promoters, e.g. Trp). It will

be appreciated that the selection of a promoter and regulatory sequences (if any) are matters of choice for those of skill in the art:

(b) host cells modified by incorporating constructs as defined in (a) immediately above so that said DNA sequences may be expressed in said host cells - the choice of host is not critical, and chosen cells may be prokaryotic or eukaryotic and may be genetically modified to incorporate said constructs by methods known in the art; and,

(c) a process for the preparation of factors as defined above comprising cultivating the modified host cells under conditions permitting expression of the DNA sequences. These conditions can be readily determined, for any particular embodiment, by those of skill in the art of recombinant DNA technology. Glial cell mitogens prepared by this means are included in the present invention.

None of the factors described in the art has the combination of characteristics possessed by the present new polypeptide factors.

The invention also includes a neuregulin as defined above, by extracting vertebrate brain material to obtain protein, subjecting the resulting extract to chromatographic purification by hydroxyapatite HPLC and then subjecting these fractions to SDS-polyacrylamide gel electrophoresis. The fraction which as an observed molecular weight of about 30 kD to 36 kD and/or the fraction which has an observed molecular weight of about 55 kD to 63 kD is collected. In either case, the fraction is subjected to SDS-polyacrylamide gel electrophoresis using the following molecular weight standards:

Lysozyme (hen egg white)	14,400
Soybean trypsin inhibitor	21,500
Carbonic anhydrase (bovine)	31,000
Ovalbumin (hen egg white)	45,000
Bovine serum albumin	66,200
Phosphorylase B (rabbit muscle)	97,400

In the case of the smaller molecular weight fraction, the SDS-polyacrylamide gel is run in non-reducing conditions in reducing conditions or, and in the case of the larger molecular weight fraction the gel is run under non-reducing conditions. The fractions are then tested for activity stimulating the division of rat Schwann cells against a background of fetal calf plasma.

Preferably, the above process starts by isolating a relevant fraction obtained by carboxymethyl cellulose chromatography, e.g. from bovine pituitary material. It is also preferred that hydroxyapatite HPLC, cation exchange chromatography, gel filtration, and/or reversed-phase HPLC be employed prior to the SDS-Polyacrylamide gel electrophoresis. At each stage in the process, activity may be determined using Schwann cell incorporation of radioactive iododeoxyuridine as a measure in an assay generally as described by Brockes in *Meth. Enz.* 147:217, 1987, but modified by substituting 10% FCP for 10% FCS.

Compounds can be assayed for their usefulness *in vitro* using the methods provided in the description and examples below. Following the *in vitro* demonstration of the effect of neuregulins on retinal cell function, the *in vivo* therapeutic benefit of the effect can be accomplished by the administration of neuregulins, neuregulin producing cells or DNA encoding neuregulins to a vertebrate requiring therapy.

In Vitro Assays of Neuregulin Effects on Retinal Cells

Several *in vitro* assays are used to determine which neuregulin protein(s) promote retinal cell function and which retinal cell types are affected by contacting neuregulin protein. Described below are methods for detecting the ability of a neuregulin to promote function of a retinal cell. *In vitro* assays for determining neuregulin effects on retinal cell function depend on establishing retinal cultures. A general reference on cell and tissue culture is *Cell and Tissue Culture: Laboratory Procedures* (Ed. by A. Doyle, J. B. Griffiths, and D. G. Newell, John Wiley and Sons, New York, NY, 1994). General references on the culture of neural cells and tissues are *Methods in Neurosciences, Vol. 2* (Ed. by P. M. Conn. Academic Press, Sand Diego, CA, 1990) and *Culturing Nerve Cells* (Ed. by G. Banker and K. Goslin, MIT Press, Cambridge, MA, 1991). General references of immunocytochemistry are *Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988), and *Immunocytochemistry II* (Ed. by A. C. Cuello, John Wiley and Sons, New York, NY, 1993).

The retinal cells from a vertebrate used in this invention may be cultured in a variety of media. Commercially available media such as Ham's F10(Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing retinal cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.* 58:44, 1979; Barnes and Sato, *Anal. Biochem.* 102:255, 1980; U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195 and U.S. Pat. Re. 30,985, may be used as culture media for retinal

cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, will be apparent to the ordinarily skilled artisan.

The use of retinal cell cultures to demonstrate that neuregulin promotes retinal cell function is in accordance with methods described in general terms above and further described in Pittack et al., *Devel.* 113:577, 1991. The retina is dissected from either embryonic or adult vertebrate animals and placed into Ca^{+2}/Mg^{+2}-free Hepes-buffered sterile saline (HBSS) for 15 min., followed by treatment with 0.25% trypsin for an additional 15 min. The trypsin is inactivated by the addition of 1% fetal bovine serum. The cells are subsequently resuspended in fresh medium and gently triturated to yield a single-cell suspension. Cells are plated into wells of 24-well plates and cultured at 37°C The types of retinal cells present in the culture can be identified through the use of immunocytochemical markers. Specific molecular markers can be stained immunocytochemically for the identification of cell types in the retina: for photoreceptors -- e.g., rhodopsin, and red and green cone opsins; for amacrine cells -- e.g., cellular retinoic acid binding protein; for bipolar cells -- e.g., a specific form of protein kinase C and its substrate protein PCP2; for retinal ganglion cells--Thy1 and ß3-tubulin and; for horizontal cells--ß3-tubulin. After maintaining the cultures for varying periods of time, preferably greater than 1 day and less than 7 days, a variety of assays can be utilized to assess various aspects of cellular phenotype such as, but not limited to, cell survival, proliferation, differentiation, morphology, and production of enzymes and secreted products.

***In Vitro* Method I**

The survival function is assayed by methods that identify and count either viable cells or dead retinal cells following culture at low density (e.g., for retinal ganglion cells 10,000 cells/cm^2) over a period from one to six days in the presence of varying amounts of neuregulin added to the culture medium. Included in these methods are specific stains for dead cells, such as propidium iodide, which enters the nucleus of dead cells and is detected

by fluorescence microscopy. Alternatively, the counting of retinal cells adhering to the culture substratum over a six day period also can be used as an indicator of cell survival.

***In Vitro* Method II**

An alternative procedure to monitor retinal cell death utilizes labeling of nicked DNA strands, which are characteristic of cells undergoing apoptotic cell death, with digoxygenin-11-dUTP using terminal deoxynucleotidyl transferase (TUNEL) according to the protocol described in Gavrieli et al., *J. Cell Biol.* 119: 493-501, 1992. The labeled DNA strands are detected using standard kits available from commercial vendors (e.g., Genius kit from Boehringer Mannheim). Further, a cell death detection ELISA system, which is based on the DNA fragmentation that occurs in dying cells (Boehringer Mannheim catalog no. 1585 045) can be utilized to quantify cell death in accordance with the instructions provided by the commercial vendor.

***In Vitro* Method III**

The release into the culture medium of the cytosolic enzyme lactate dehydrogenase (LDH) also can be used to quantify the extent of retinal cell death *in vitro* (Kirk et al, *J. Pharmacol. Exper. Therapeut.* 271:1080, 1994). LDH levels are measured by an automated kinetic colorimetric assay in which oxidation of lactate to pyruvate is coupled to reduction of the tetrazolium dye, INT. Briefly, 80 ul samples of the culture medium are mixed with an equal volume of the substrate solution containing (in mg/l) INT, 334; phenazine methosulfate, 86; nicotinamide adenine dinucleotide, 862; L-(+)-lactate, 4900 (lithium salt); and 0.1% Triton X-100 in 0.2 M Tris buffer, pH 8.2. In the assay, LDH activity is directly proportional to the rate of appearance of the resulting INT formazan (absorbance max. at 492 nm). The product is monitored quantitatively in a microplate reader (UVmax, Molecular Devices, Menlo Park, CA) as the change in absorbance at 490 nm over a 2 min. interval.

***In Vitro* Method IV**

The proliferative function of neuregulins on retinal cells can be assayed by incorporation of ^{125}I-Urd, ^{3}H-dT or BrdU into replicating DNA strands of dividing cells, or by cell counting. The assays developed to measure the mitogenic activity of neuregulins on Schwann cells by incorporation of DNA synthesis precursors (Brockes et al., *Brain Res.*

165:105, 1979; Davis and Stroobant, *J. Cell Biol.* 110: 1353, 1990) can be adapted to retinal cells by one of normal skill in the art of cell culture.

***In Vitro* Method V**

The differentiation function of neuregulin on retinal cells can be assayed by employing analytical methods, such as immunostaining or *in situ* hybridization, which can detect and quantify marker proteins associated with the various cell types of the retina. Retinal ganglion cells are recognized by staining with the specific tubulin antibody TUJ1, as shown in Example 2 (Figure 4). The glial cells of the retina, Müller glia, are recognized by staining with antibodies that recognize glial fibrillary acidic protein (GFAP). For example, neurogenesis of retinal cells in culture can be achieved by dissociating embryonic retinal progenitor cells of the rat (from E15 through E18), then contacting the cells with the neuregulin and quantifying the distribution of various cell types identified by immunostaining using the markers described herein. In addition to this assay, which is based on determining activity in retinal neurogenesis, the differentiation function of neuregulin can be assayed in mature cultures (e.g., differentiated in culture for approximately two weeks). As such, changes in the level of specific proteins expressed in particular retinal cell types can be quantified.

***In Vitro* Method VI**

Several peptide growth factors and their receptors have been identified in the retina, as described in the prior act. Methods utilized to detect those molecules and activities can be employed to demonstrate a differentiation function of neuregulin on retinal cells. Neuregulins can be shown to induce the synthesis of growth factors and/or their receptors expressed in the retina. The analysis can be by *in situ* hybridization or other methods of quantitative RNA analysis, such as, but not limited to, reverse transcription-PCR, RNAse protection and Northern blotting. Alternatively, induced expression of growth factors or their receptors can be assayed by immunocytochemical staining or cell biological assays designed to measure growth factor activity.

The *in vitro* assays described above to identify neuregulins that have biological activity on retinal cells can be applied to dissociated cells, semi-dissociated cells, explants of whole retina and parts thereof, such as preparations of retinal pigmented epithelium and other layers of the retina. The cultures can be established and maintained using methods described above. In some cases, minor modifications or substitutions to the procedures

described herein, which do not alter the reduction to practice of the invention, can be provided by one of ordinary skill in the art.

In Vivo Assays of Neuregulin Effects on Retinal Cells

Neuregulin activity on retinal cells also can be shown through *in vivo* assays. Some *in vivo* assays represent animal models of retinal degeneration and other diseases and disorders of the eye. For example, photoreceptor cells are lost in inherited retinal degeneration and in age-related macular degeneration. Retinal ganglion cells die in glaucoma and in optic nerve injuries, such as retinal ischemia or axotomy.

In Vivo Method I

The rescue of photoreceptor cells can be demonstrated in Royal College of Surgeons (RCS) rats, which have an inherited retinal degeneration (Faktorovich et al., *Nature* 347:83, 1990). The histological analysis (Method H1) consists of vascular perfusion of anesthetized animals, embedding the eye in epoxy resin, then staining 1 micron sections with toluidine blue. In untreated RCS rats at 53 days after birth (P53) the outer nuclear layer, which contains the photoreceptor cells, is reduced in thickness to only a few rows of cells (approximately 20% of the thickness found in normal rats at the same age). A therapeutically effective dose of neuregulin administered by intravitreal administration (a single injection of 1 microliter) can restore the thickness of the outer nuclear layer, and hence rescue photoreceptor cells. Alternatively, rescue of photoreceptor cells can be demonstrated in the Sprague-Dawley rat models (2-to-3 month old males) of exposure to constant light (115-200 foot-candles) for 1 week (LaVail et al., *PNAS USA* 89:11249, 1992). Neuregulin can be injected (1 ul) into the subretinal space or into the vitreous humor 48 hours prior to the onset of continuous illumination. Histological analysis (Method H1) of retinas following a fixed recovery period (usually 10 days) is used to assess the damage to and rescue of photoreceptor cells. Retinal detachment also leads to the death of photoreceptor cells, which provides another animal model (Erickson et al., *J. Struct. Biol.* 108:148, 1992) to demonstrate the *in vivo* survival activity of neuregulin on retinal cells.

In Vivo Method II

Several mouse genetic models of photoreceptor degeneration (e.g., *rd*--mutant of b subunit of cGMP phosphodiesterase; *rds*--mutant of peripherin) can be used to show neuregulin survival effects *in vivo* using the modes of administration described above. The *rd* and *rds* animals show retinal degeneration within a few weeks after birth and following intravitreal injection of neuregulin tissues can be analyzed by histological methods described above (e.g., Method H1). Further, retinal explants from *rd* mice cultured in neuregulin-containing medium can be assayed for thickness of the outer nuclear layer using methods described in Caffe et al., *Curr. Eye Res.* 12:719, 1993. Mouse pups are enucleated 48 hours after birth and treated with proteinase K. After enzyme treatment, the neural retina with the retinal pigmented epithelium (RPE) attached is recovered, placed into a multi-well culture dish and incubated in 1.2 ml culture medium (e.g., R16) for up to 4 weeks at 37 C with 5 % CO_2. Immunocytochemical staining for opsin of fixed (e.g., 4% paraformaldehyde) sections is used to assess the degeneration and rescue of photoreceptor cells. In the *rd* mouse the outer nuclear layer (photoreceptor cells) degenerate after 2-to-4 weeks in culture. The

media can be supplemented with varying doses of neuregulin to achieve an effect on retinal cell function, such as rescue of the outer nuclear layer from degeneration. Survival effects also can be shown using the TUNEL method on sections of retina analyzed in the models described above.

***In Vivo* Method III**

In response to injury to the retina Müller cells undergo a proliferative gliosis. The mitotic activity of neuregulin on Müller glia can be shown by labeling dividing cells with a DNA synthesis precursor following administration of the factor. Labeled cells can be detected by autoradiography (^{3}H-dT) or by immunostaining (BrdU labeling) and quantified.

***In Vivo* Method IV**

Neuregulins can be shown to promote retinal ganglion cell survival following optic nerve axotomy or nerve crush using methods described in Sievers et al., *Neurosci. Lett.* 76:157, 1987; Carmignoto et al., *J. Neurosci.* 9:1263, 1989; Mey and Thanos, *Brain Res.* 602:304, 1993. Briefly, 4-to-6 week old mice are anesthetized, the optic nerve exposed and crushed intraorbitally 2-4 mm posterior to the optic disk between fine forceps for 30-60 sec. Alternatively, the nerve is transected surgically. Administration of neuregulin by intravitreal or subretinal injection is done after the animals recover from surgery using a therapeutically effective dose. The survival of retinal ganglion cells is assessed at several time points between 3 days and 6 weeks after injection by histological analysis (Method H1) or by immunostaining using antibodies that recognize retinal ganglion cells as described herein.

***In Vivo* Method V**

Ischemia can be produced in the retina of the albino Lewis rat by raising intraocular pressure by intraocular injection of saline (Unoki and LaVail, *Invest Ophthalmol Vis. Sci.* 35:907, 1994). The thickness of the inner retinal layer is reduced due to loss of retinal ganglion cells when retinas are analyzed histologically (Method H1) at 7 days post-ischemia. An intravitreal injection of a therapeutically effective amount of neuregulin given two days prior to ischemia can reduce the ischemic damage.

***In Vivo* Method VI**

Other compounds, in particular peptides, which specifically bind and/or activate erbB receptors also can be used according to the invention as effectors of retinal cell

function. A candidate compound can be routinely screened for erbB receptor binding, and if it binds, can then be screened for affecting retinal cell function using the methods described herein.

In Vivo Method VII

Inadequate amounts of survival-promoting factors can lead to degenerative eye disorders, such as macular degeneration. The present invention demonstrates the survival-promoting activity of neuregulin indicating that these factors may be used to promote retinal cell survival in a vertebrate (preferably a mammal, more preferably a human) by administering to the vertebrate an effective amount of a polypeptide or a related compound. Neuregulin effects on retinal cells may occur, for example, by preventing the extent of naturally-occurring programmed cell death that occurs during the embryonic development of the retina. In a rat model, retinal ischemia can be induced by increasing intraocular pressure via injection of saline into the eye (Buchi et al., *Ophthalmologic.* 203:138, 1991; Hughes, *Exp. Eye Res.* 53:573, 1991). This model has been used to evaluate the efficacy of bFGF, CNTF and BDNF in decreasing neuronal loss (Unoki and LaVail, *Invest. Ophthalmol. Vis. Sci.* 35:907, 1994). Neuregulins administered by intraocular injection can be shown to decrease neuronal loss associated with retinal ischemia in this animal model. Neuregulin effects on retinal cell survival can be shown in genetic and transgenic mouse models for Retinitis Pigmentosa (*rp*). Histological analysis (Method H1) of the retina of *rp* mice following intravitreal administration of neuregulin can be used to rescue retinal cell degeneration.

The demonstration of biological activity of the neuregulins by promoting retinal cell function in any of the animal models described above indicates efficacy in treating disorders of the eye. A variety of retinal diseases and related disorders are known that produce impaired vision and in some cases progress to total blindness. These disorders of the eye include, but are not necessarily limited to: various retinopathies, such as hypertensive retinopathy, diabetic retinopathy and occlusive retinopathy; also injuries and disorders resulting in retinal degeneration, such as retinal tearing and detachment and inherited diseases, such as retinitis pigmentosa; also age-related macular degeneration (ARMD) and related diseases, such as idiopathic central serous chorioretinopathy, central areolar choroidal dystrophy, macular holes, macular coloboma, Stargardt hereditary dystrophy, trauma, diabetic circinate maculopathy, angioid streaks and choroidal neovascularization, presumed ocular histoplasmosis and choroidal neovascularization, angiomatosis retinae, choroidal rupture and choroidal neovascularization, toxoplasmosis and choroidal

neovascularization; diseases.of the optic nerve; and glaucoma and retinal ischemia. Thus, administration of neuregulin in a therapeutically effective amount can provide a treatment for disorders of the eye, which otherwise left untreated would result in the loss of sight.

The invention includes the use of any modifications or equivalents of the above polypeptide factors which do not exhibit a significantly reduced activity related to affecting retinal cell function. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting activity are included. The statements of effect and use contained herein are therefore to be construed accordingly, with such uses and effects employing modified or equivalent factors being part of the invention.

The invention includes the use of the above named family of proteins (i.e. neuregulins) as extracted from natural sources (tissues or cell lines) or as prepared by recombinant means.

The human peptide sequences described above represent a series of splicing variants which can be isolated as full length complementary DNAs (cDNAs) from natural sources (cDNA libraries prepared from the appropriate tissues) or can be assembled as DNA constructs with individual exons (e.g., derived as separate exons) by someone skilled in the art.

The invention includes methods for the use of any protein which is substantially homologous to the coding segments in Figure 13, as well as other naturally occurring neuregulin polypeptides for the purpose of promoting retinal cell function. Also included are the use of: allelic variations; natural mutants; induced mutants; proteins encoded by DNA that hybridizes under high or low stringency conditions to a nucleic acid naturally occurring (for definitions of high and low stringency see *Current Protocols in Molecular Biology*, (1989) John Wiley & Sons, New York, NY, 6.3.1 - 6.3.6, hereby incorporated by reference); and the use of polypeptides or proteins specifically bound by antisera to GGF polypeptides. The term also includes the use of chimeric polypeptides that include the GGF polypeptides comprising sequences from Figure 13.

Use of Neuregulins

A novel aspect of the invention involves the use of neuregulins as factors to promote retinal cell function. Treatment of the cells to achieve these effects may be achieved by contacting cells with a polypeptide described herein.

The methods of the invention make use of the fact that the neuregulin proteins are encoded by the same gene. A variety of messenger RNA splicing variants (and their resultant proteins) are derived from this gene and many of these products show binding to erbB receptors and activation of the same. This invention provides a use for all of the known products of the neuregulin gene (described herein and in the references listed above). Most preferably, recombinant human GGF2 (rhGGF2) is used in these methods.

The invention also relates to the use of other, not yet naturally isolated, splicing variants of the neuregulin gene. Figure 12 shows the known patterns of splicing. These patterns are derived from polymerase chain reaction experiments (on reverse transcribed RNA), analysis of cDNA clones (as presented within), and analysis of published sequences encoding neuregulins (Peles et al., *Cell* 69:205, 1992 and Wen et al., *Cell* 69:559, 1992). These patterns, as well as additional patterns disclosed herein, represent probable splicing variants which exist. The splicing variants are fully described in Goodearl et al., USSN 08/036,555, filed March 24, 1993, incorporated herein by reference.

More specifically, effects on retinal cell function may be achieved by contacting cells with a polypeptide defined by the formula

WYBAZCX

wherein WYBAZCX is composed of the polypeptide segments shown in Figure 13; wherein W comprises the polypeptide segment F, or is absent; wherein Y comprises the polypeptide segment E, or is absent; wherein Z comprises the polypeptide segment G, or is absent; and wherein X comprises the polypeptide segment C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' D' HKL.

Furthermore, the invention includes a method of treating retinal cells by the application to the retinal cell of a

-30 kD polypeptide factor isolated from the MDA-MB 231 human breast cell line; or

-35 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line to the glial cell; or

-75 kD polypeptide factor isolated from SKBR-3 human breast cell line; or

-44 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line; or

-25 kD polypeptide factor isolated from activated mouse peritoneal macrophages; or

-45 kD polypeptide factor isolated from the MDA-MB 231 human breast cell; or

-7 to 14 kD polypeptide factor isolated from the ATL-2 human T-cell line to the glial cell; or

-25 kD polypeptide factor isolated from the bovine kidney cells; or

-42 kD ARIA polypeptide factor isolated from brain; or

-46-47 kD polypeptide factor which stimulates 0-2A glial progenitor cells; or

-43-45 kD polypeptide factor. GGFIII. U.S. patent application Serial No. 07/931,041. filed August 17. 1992, incorporated herein by reference.

The invention includes use of any modifications or equivalents of the above polypeptide factors which do not exhibit a significantly reduced activity. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting activity are included. The statements of effect and use contained herein are therefore to be construed accordingly, with such uses and effects employing modified or equivalent factors being part of the invention.

The human peptide sequences described above and presented in Figs. 13, 14, 15, and 16, respectively, represent a series of splicing variants which can be isolated as full-length complementary DNAs (cDNAs) from natural sources (cDNA libraries prepared from the appropriate tissues) or can be assembled as DNA constructs with individual exons (e.g., derived as separate exons) by someone skilled in the art.

Another aspect of the invention is the use of a pharmaceutical or veterinary formulation comprising any factor as defined above formulated for pharmaceutical or veterinary use, respectively, optionally together with an acceptable diluent, carrier or excipient and/or in unit dosage form. In using the factors of the invention, conventional pharmaceutical or veterinary practice may be employed to provide suitable formulations or compositions.

A medicament is made by administering the polypeptide with a pharmaceutically effective carrier. Neuregulins can be administered intravitreally by insertion of a needle through the sclera, choroid and retina and then injection of factor formulated in an appropriate vehicle for administration. The factor may also be delivered subretinally by a transpleural injection. There is also the option of delivering the factor intraocularly using ethylene-vinyl acetate copolymer implants or by delivery to the corneal surface via eye drops.

Thus, the formulations to be used as a part of the invention can be applied to parenteral administration, for example, intravenous, subcutaneous, intramuscular, intraorbital, ophthalmic, intraperitoneal, topical, intranasal, aerosol, transdermal and by other slow release devices (i.e., osmotic pump-driven devices; see also USSN 08/293,465, hereby incorporated by reference).

The formulations of this invention may also be administered by the transplantation into the patient of host cells expressing the DNA encoding polypeptides which are effective for the methods of the invention or by the use of surgical implants which release the formulations of the invention.

Parenteral formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations maybe in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well-known in the art for making formulations are to be found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain as excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes, biocompatible, biodegradable lactide polymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the present factors. Other potentially useful parenteral delivery systems for the factors include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal administration.

The present factors can be used as the sole active agents, or can be used in combination with other active ingredients, e.g., other growth factors which could facilitate neuronal survival in neurological diseases, or peptidase or protease inhibitors.

The concentration of the present factors in the formulations of the invention will vary depending upon a number of issues, including the dosage to be administered, and the route of administration.

In general terms, the factors of this invention may be provided in an aqueous physiological buffer solution containing about 0.1 to 10% w/v compound for parenteral administration. General dose ranges are from about 1 µg/kg to about 1g/kg of body weight per day; a preferred dose range is from about 0.01 mg/kg to 100 mg/kg of body weight per day. The preferred dosage to be administered is likely to depend upon the type and extent of progression of the pathophysiological condition being addressed, the overall health of the patient, the make up of the formulation, and the route of administration.

A further general aspect of the invention is the use of a factor of the invention in the manufacture of a medicament, preferably for the treatment of a disease or disorder. The "GGF2" designation is used for all clones which were previously isolated with peptide sequence data derived from GGF-II protein (i.e., GGF2HBS5, GGF2BPP3) and, when present alone (i.e., GGF2 OR rhGGF2), to indicate recombinant human protein encoded by plasmids isolated with peptide sequence data derived from the GGF-II protein (i.e., as produced in insect cells from the plasmid HBS5). Recombinant human GGF from the GGFHBS5 clone is called GGF2, rhGGF2 and GGF2HBS5 polypeptide.

Methods for treatment of diseases or disorders using nucleic acid constructs encoding neuregulins or neuregulin producer cells are also part of the invention.

Delivery of DNA to a cell or tissue that will take up the DNA, express the DNA and produce neuregulin as shown by Wolff et al., (*Science* 247:1465, 1990) and Ascadi et al., (*Nature* 352:815, 1991) is an aspect of the invention. Genetic modification of cultured cells (or their precursors) such as fibroblasts (as shown by Wolff et al. *Proc. Nat'l Acad. Sci. USA* 86:1575, 1988) or such as those derived from the nervous system (as shown by Weiss et al. International Patent Application number PCT/US94/01053; publication number WO 94/16718) to induce the production of neuregulin from the cultured cells is another aspect of this invention. The genetically modified neuregulin producer cells can be transplanted to a position near the retinal cell type and elicit the responses described above.

Other Embodiments

The invention includes methods for the use of any protein which is substantially homologous to the coding segments in Figure 13 as well as other naturally occurring GGF or neuregulin polypeptides for the purpose of promoting retinal cell function. Also included are the use of: allelic variations: natural mutants; induced mutants; proteins encoded by

DNA that hybridizes under high or low stringency conditions to a nucleic acid naturally occurring (for definitions of high and low stringency see *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1989; 6.3.1-6.3.6, hereby incorporated by reference); and the use of polypeptides or proteins specifically bound by antisera to GGF polypeptides. The term also includes the use of chimeric polypeptides that include the GGF polypeptides comprising sequences from Figure 11 for the promotion of retinal cell function.

As will be seen from Example 2, below, the present factors exhibit survival activity on retinal cells. The general statements of invention above in relation to formulations and/or medicaments and their manufacture should clearly be construed to include appropriate products and uses.

A series of experiments follow which provide additional basis for the claims described herein. The following examples relating to the present invention should not be construed as specifically limiting the invention, or such variations of the invention, now known or later developed.

The examples illustrate our discovery that recombinant human GGF2 (rhGGF2) confers survival effects on retinal cell culture. These activities indicate efficacy of GGF2 and other neuregulins in inducing wound repair and repair of other retinal tissue damage, and promoting regeneration and prophylactic effects on retinal tissue degeneration.

EXAMPLES

The following examples are designed to illustrate certain aspects of the present invention. The examples are not intended to be comprehensive of all embodiments of the present invention. and should not be construed as limiting the claims presented herein.

Example 1: Neuregulin expression in embryonic and adult retina.

Neuregulin is expressed in the retina of the developing embryo and in adult rat. The pattern of expression has been demonstrated by *in situ* hybridization (Figure 3) and also by immunostaining (Figures 2 and 4). Expression is detected in the retinal ganglion cell layer. The expression occurs at a point in development when the retinal layers are undergoing differentiation and when the retinal ganglion cells are extending their axons and making connections to target of innervation in the brain (lateral geniculate and superior colliculus). The timing and distribution of neuregulin gene products in the retina suggests the neuregulins have a role in the development and/or maintenance of the cells in the retina and their associated tissues.

Methods

In situ hybridization (see Figure 3). Ten micron frozen section was incubated with a single-stranded digoxigenin-labeled riboprobe (antisense strand) encoding the EGF-like domain through the cytoplasmin domain of the rat cDNA clone GGFRP3 (Marchionni et al., *Nature* 362:312. 1993).

Immunostaining. Ten micron frozen section of embryonic day 16 rat retina was incubated in CN16 (anti-rhGGF2) antibody at approximately 10 mg/ml for 12 hours, and the antibody binding was revealed using indirect immunohistochemistry with a peroxidase conjugated secondary antibody (see Figure 2).

Ten micron frozen section from an adult rat retina was incubated in CN16 as described in Figure 1, except that a fluorescein conjugated secondary antibody was used to reveal the binding of the primary antibody (see Fig 4). Neuregulin immunoreactivity is present in the synaptic layers of the retina, where the processes of the retinal ganglion cells connect with the retinal interneurons (inner plexiform layer, large arrows) and in the outer plexiform layer (small arrows, where the processes of the photoreceptors make synapses with the second order retinal neurons, the bipolar cells and the horizontal cells).

Ten micron section from a newborn rat retina incubated with TUJ1 antibody, a mouse monoclonal antibody that recognizes neuron-specific beta -tubulin (from Dr. A. Frankfurter, UVA). The antibody binding was revealed by indirect immunohistochemistry with a fluorescein conjugated secondary antibody (see Figure 5).

Example 2: Neuregulin (rhGGF2) promotes survival of retinal cells in vitro.

Embryonic and newborn rat retinal cells were cultured for 2 days on collagen gel coated coverslips and then fixed and labeled with an antibody (TUJ1) that identifies primarily retinal ganglion cells at these stages of development (see Figure 6). All the labeled cells with processes on the sample coverslips were counted. Final concentrations of rhGGF2 in the culture wells ranged from 0.01 to 100 ng/ml. No clear dose response was observed, so the data from all rhGGF2 treated wells was combined for the analysis. Three separate experiments with embryonic day 18 cells all showed an increase in the number of TUJ1 immunoreactive cells with processes after two days *in vitro* (see Figure 7). Unpaired sample student's T-test showed that the increases in cell survival were statistically significant with $p<0.004$ and $p<0.012$. Two experiments with embryonic day 15 cells and one experiment with newborn rat retinal cells did not show any significant differences from control (see Figure 8). From these observations we conclude that rhGGF2 promotes rat retinal cell survival in cell culture in an age-dependent manner. This age-dependence could represent either a changing requirement for this factor of a specific retinal cell population or a change in the relative number of the responsive cells in the population during these developmental stages. When assayed either alone or in combination with EGF, rhGGF2 had no mitogenic activity retinal cells *in vitro* at any of the ages tested.

Methods

Embryonic rat retinal cell were dissociated and plated at low density on collagen gels and allowed to survive for two days with 10 ng/ml rhGGF2 (neuregulin) added to the medium. After fixation in 4% paraformaldehyde, the cells were stained with TUJ1 antibody to reveal the full extent of their processes and all the cells that survived for two days with intact, non-fragmented processes were counted.

CLAIMS

What is claimed is:

1. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide defined by the formula

WYBAZCX

wherein WYBAZCX is composed of the polypeptide segments shown in Figure 13; wherein W comprises polypeptide segment F, or is absent; wherein Y comprises polypeptide segment E, or is absent; wherein Z comprises polypeptide segment G, or is absent; and wherein X comprises polypeptide segments C/D HKL, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' D' HKL.

2. A method of claim 1, wherein X is C/D HKL.

3. A method of claim 1, wherein X is C/D H.

4. A method of claim 1, wherein X is C/D HL.

5. A method of claim 1, wherein X is C/D D.

6. A method of claim 1, wherein X is C/D' HL.

7. A method of claim 1, wherein X is C/D' HKL.

8. A method of claim 1, wherein X is C/D' H.

9. A method of claim 1, wherein X is C/D' D.

10. A method of claim 1, wherein X is C/D C/D' HKL.

11. A method of claim 1, wherein X is C/D C/D' H.

12. A method of claim 1, wherein X is C/D C/D' HL.

13. A method of claim 1, wherein X is C/D C/D' D.

14. A method of claim 1, wherein X is C/D D' H.

15. A method of claim 1, wherein X is C/D D' HL.

16. A method of claim 1, wherein X is C/D D' HKL.

17. A method of claim 1, wherein X is C/D' D' H.

18. A method of claim 1, wherein X is C/D' D' HL.

19. A method of claim 1, wherein X is C/D' D' HKL.

20. A method of claim 1, wherein X is C/D C/D' D' H.

21. A method of claim 1, wherein X is C/D C/D' D' HL.

22. A method of claim 1, wherein X is C/D C/D' D' HKL.

23. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising FBA polypeptide segments having the amino acid sequences shown in Figure 11.

24. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising FBA' polypeptide segments having the amino acid sequences shown in Figure 11.

25. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising FEBA polypeptide segments having the amino acid sequences shown in Figure 11.

26. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising FEBA' polypeptide segments having the amino acid sequences shown in Figure 11 to said retinal cells.

27. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with GGF2 polypeptide.

28. The method of claim 27, wherein said GGF2 is recombinant human GGF2.

29. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a compound which binds with erbB receptors of said retinal cells.

30. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising EGFL1, having the amino acid sequence shown in Figure 18.

31. A method of treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising EGFL2, having the amino acid sequence shown in Figure 19.

32. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising EGFL3, having the amino acid sequence shown in Figure 20.

33. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising EGFL4, having the amino acid sequence shown in Figure 21.

34. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising EGFL5, having the amino acid sequence shown in Figure 22.

35. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising EGFL6, having the amino acid sequence shown in Figure 23.

36. A method for treating retinal cells of a mammal, said method comprising contacting a 45 kD polypeptide factor isolated from the rat I-EJ *ras*-transformed fibroblast cell line to said retinal cells.

37. A method for treating retinal cells of a mammal, said method comprising contacting a 75 kD polypeptide factor isolated from SKBR-3 human breast cell line to said retinal cells.

38. A method for treating retinal cells of a mammal, said method comprising contacting a 45 kD polypeptide factor isolated from the MDA-MB231 human breast cell line to said retinal cells.

39. A method for treating retinal cells of a mammal, said method comprising contacting a 7 to 14 kD polypeptide factor isolated from the ATL-2 human T-cell line to said retinal cells.

40. A method for treating retinal cells of a mammal, said method comprising contacting a 25 kD polypeptide factor isolated from activated mouse peritoneal macrophages to said retinal cells.

41. A method for treating retinal cells of a mammal, said method comprising contacting a 25 kD polypeptide factor isolated from bovine kidney to said retinal cells.

42. A method for treating retinal cells of a mammal, said method comprising contacting an ARIA polypeptide to said retinal cells.

43. A method for treating retinal cells of a mammal, said method comprising contacting a 46-47 kD polypeptide factor known to stimulate 0-2A glial progenitor cells to said retinal cells.

44. A method for treating retinal cells of a mammal, said method comprising contacting GGF-III to said retinal cells.

45. A method for treating retinal cells of a mammal, said method comprising administration to said mammal of a DNA sequence encoding a polypeptide of the formula

WYBAZCX

wherein WYBAZCX is composed of the polypeptide segments shown in Figure 13; wherein W comprises polypeptide segment F, or is absent; wherein Y comprises polypeptide segment E, or is absent; wherein Z comprises polypeptide segment G, or

is absent; and wherein X comprises polypeptide segments C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' HKL, said DNA in an expressible genetic construction.

46. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a therapeutically effective amount of a neuregulin polypeptide.

47. A method for the prophylaxis or treatment of pathophysiological condition of retinal cells in a mammal in which said condition involves a retinal cell type which is sensitive or responsive to a neuregulin polypeptide, said method comprising administration of a therapeutically effective amount of said neuregulin polypeptide.

48. A method for the treatment of a condition which involves retinal cell damage in a mammal, said method comprising contacting said retinal cell with an effective amount of a neuregulin polypeptide.

49. The method of any one of claims 1 through 28, wherein a result of said treating is decreased atrophy of said retinal cells.

50. The method on any one of the claims 1 through 28, wherein a result of said treating is an increase of said retinal cells present in said mammal.

51. The method on any one of the claims 1 through 28, wherein a result of said treating is an increase in said retinal cells survival in said mammal.

52. A method of any one of the claims 1 through 28, wherein said retinal cells are in a mammal with a retinal cell disease.

53. A method of claim 52, wherein said retinal cell disease is a retinopathy.

54. A method of claim 53, wherein said retinopathy is hypertensive retinopathy.

55. A method of claim 53, wherein said retinopathy is diabetic retinopathy.

56. A method of claim 53, wherein said retinopathy is occlusive retinopathy.

57. A method of claim 52, wherein said retinal cell disease is retinal degeneration.

58. A method of claim 57, wherein said retinal degeneration is caused by injury.

59. A method of claim 57, wherein said retinal degeneration is caused by a genetic disorder.

60. A method of claim 59, wherein said genetic disorder is retinitis pigmentosa.

61. A method of claim 57, wherein said retinal degeneration is age related macular degeneration.

62. A method of claim 52, wherein said retinal disease is caused by elevated intraocular pressure.

63. A method of claim 52, wherein said retinal disease is caused by an optic neuropathy.

64. A method for the prophylaxis or treatment of a pathophysiological condition of a retina in a vertebrate in which said condition involves a retinal cell type which is sensitive or responsive to a neuregulin polypeptide, said method comprising administration to said vertebrate of a therapeutically effective amount of said neuregulin polypeptide.

65. A method of claim 53, wherein said condition involves retinal cell damage.

66. A method of any one of claims 1 through 28, wherein said retinal cell is a retinal ganglion cell.

67. A method of any one of claims 1 through 28, wherein said retinal cell is an amacrine cell.

68. A method of any one of claims 1 through 28, wherein said retinal cell is a horizontal cell.

69. A method of any one of claims 1 through 28, wherein said retinal cell is a bipolar cell.

70. A method of any one of claims 1 through 28, wherein said retinal cell is a photoreceptor cell.

71. A method of any one of claims 1 through 28, wherein said retinal cell is a pigment cell.

72. A method of treating retinal cells of a mammal, said method comprising contacting an N-ARIA polypeptide to said retinal cells.

Figure 1

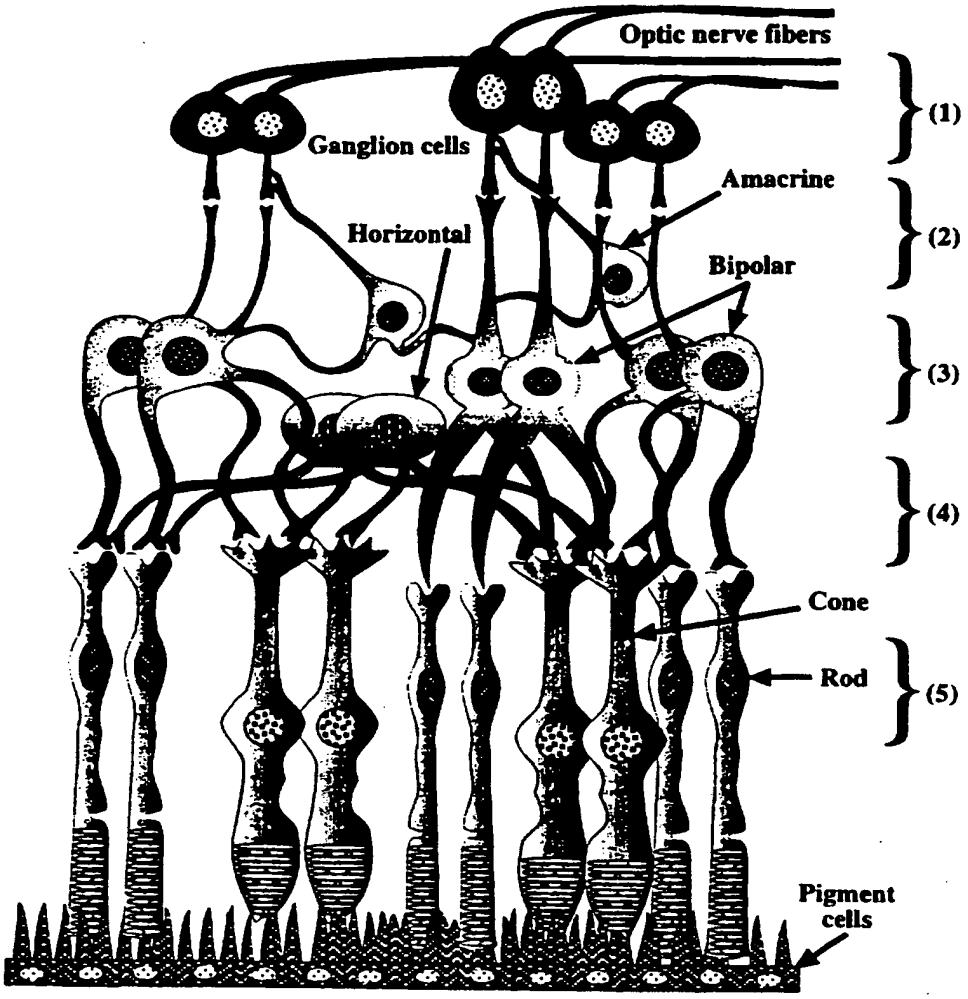

Figure 2

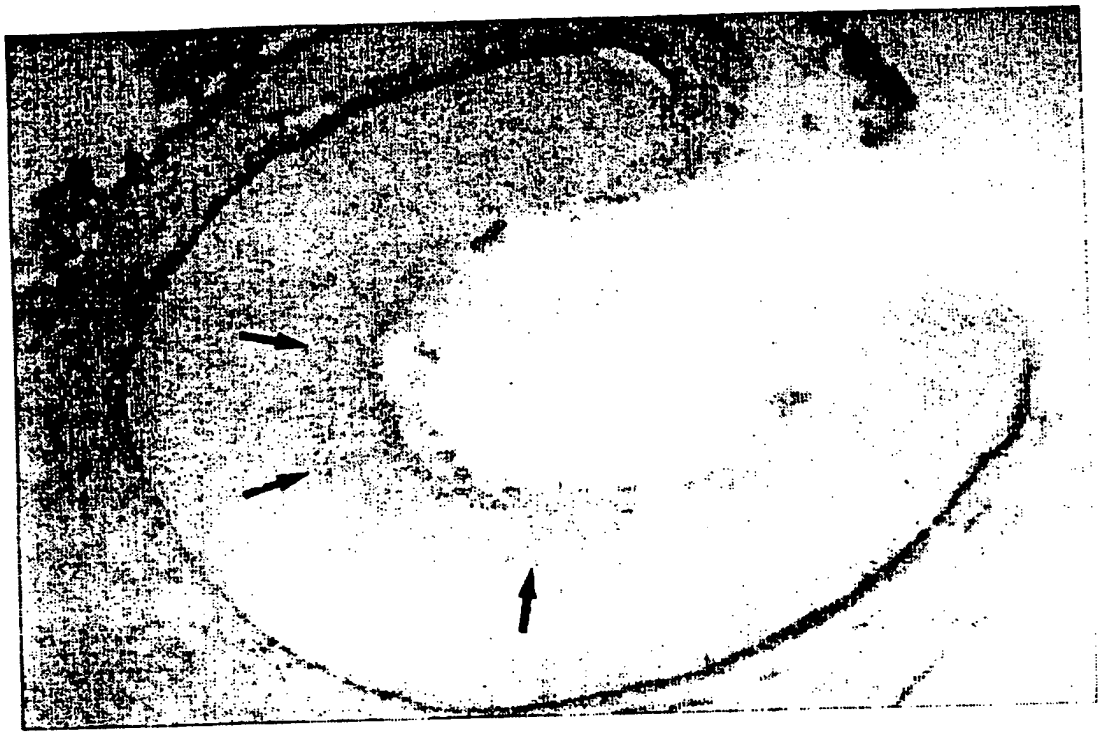

Figure 3

Figure 4

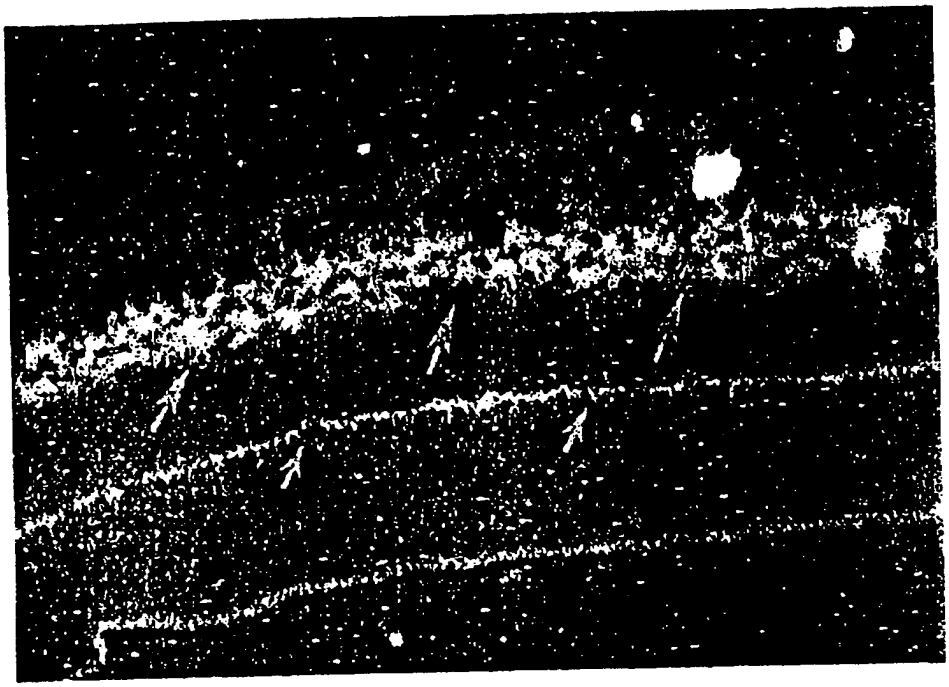

Figure 5

Figure 6

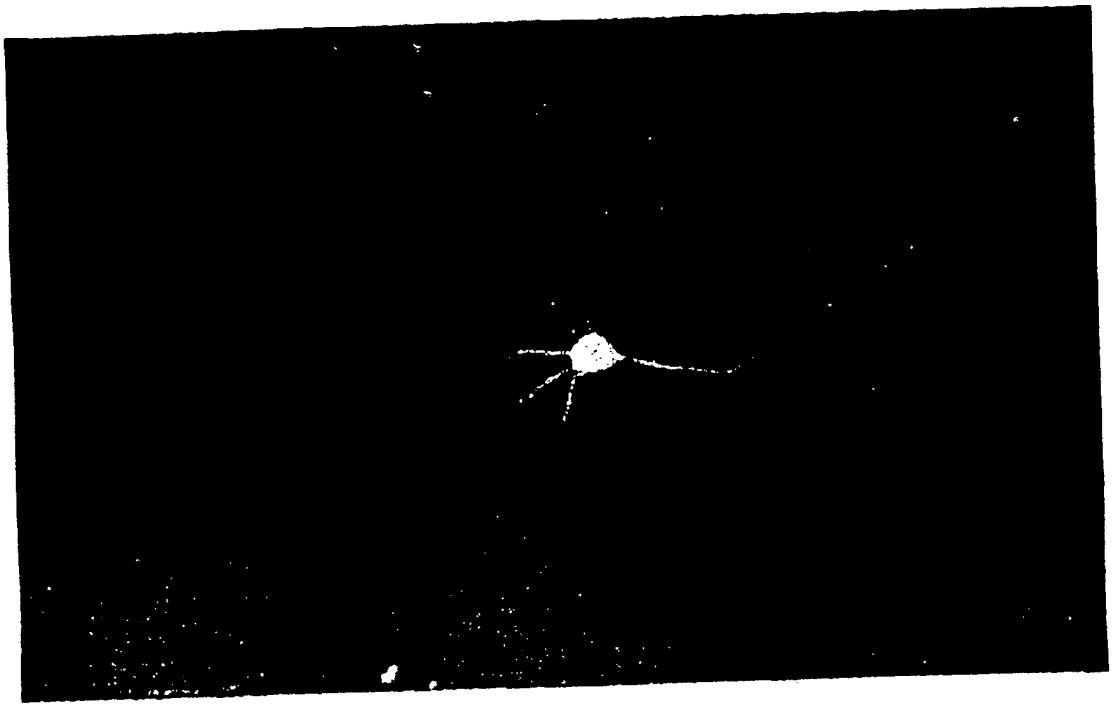

Figure 7

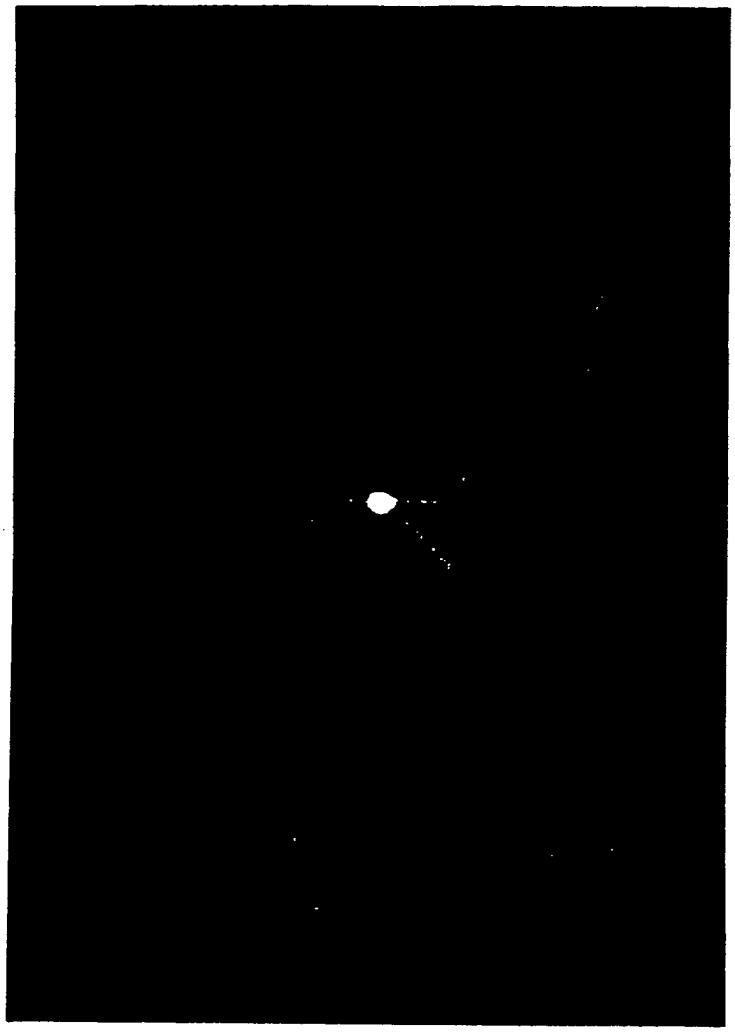

Figure 8

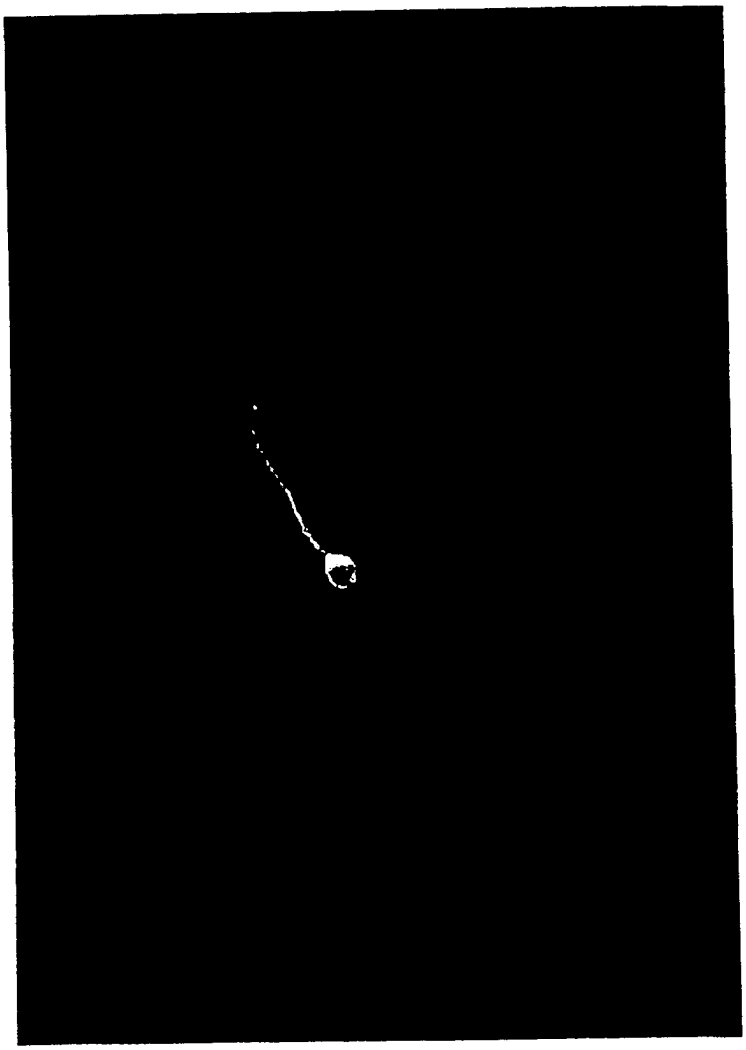

Figure 9

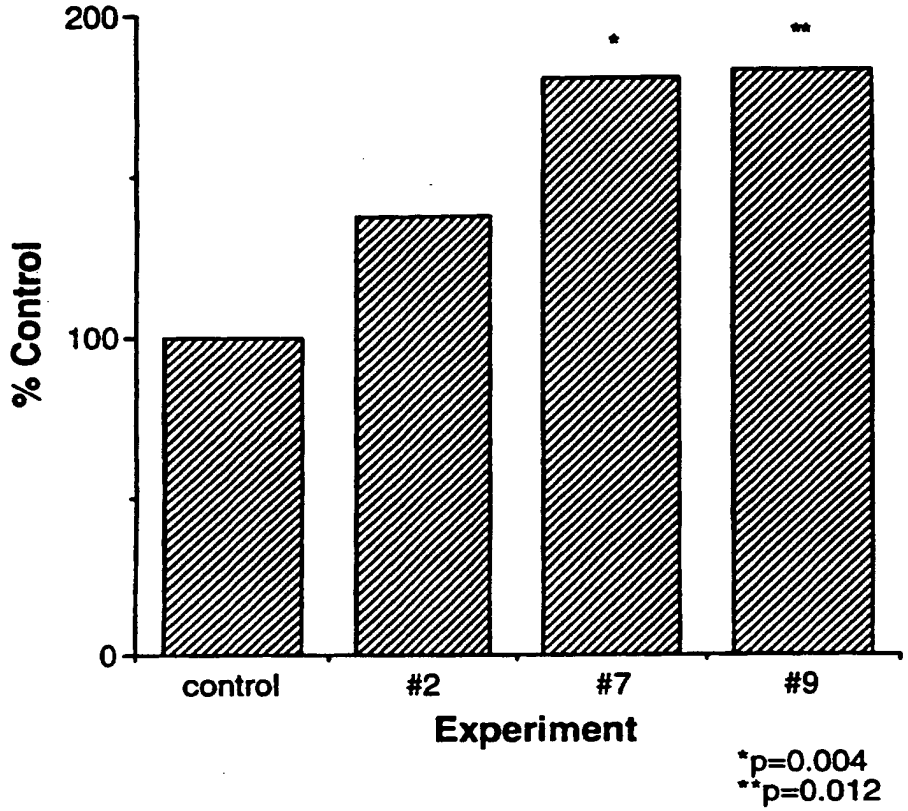

Figure 10

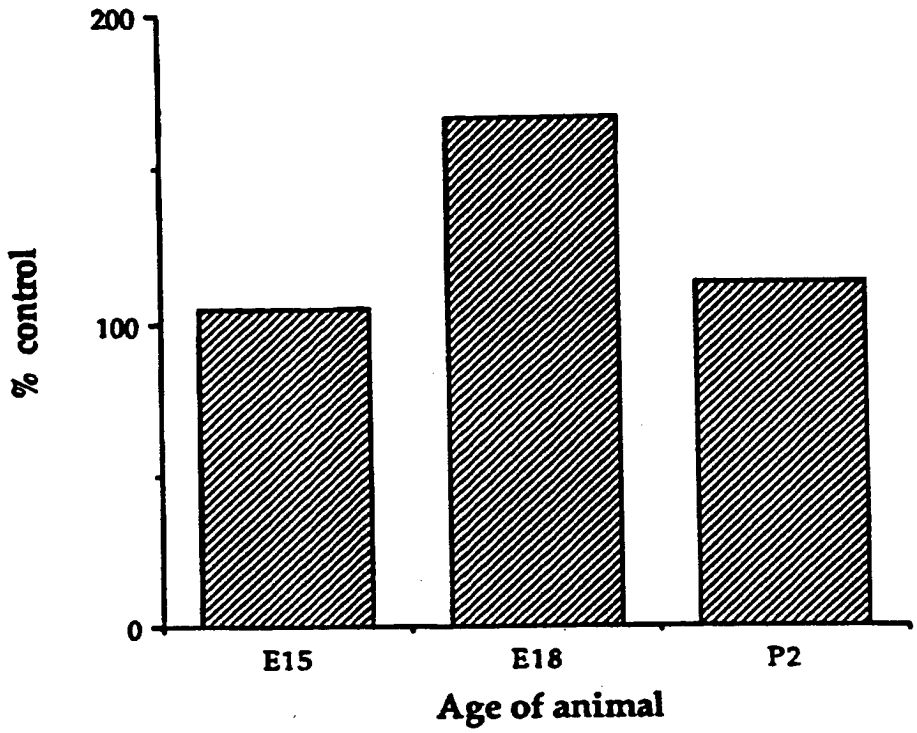

Figure 11 A

```
CCTGCAG CAT CAA GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG      55
        His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu
          1               5                  10                  15

CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC GCC TTC CCC TCC TGC          103
Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser Cys
        20                  25                  30

GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAG          151
Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro Glu
      35                  40                  45
GCC AAC AGC AGC GGC GGG CCC GGC CGC CTT CCG AGC CTC CTT CCC CCC          199
Ala Asn Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro Pro
  50                  55                  60

TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT GTG          247
Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala Val
65                  70                  75                  80

CAA CGG TGC GCC TTG CCT CCC CGC TTG AAA GAG ATG AAG AGT CAG GAG          295
Gln Arg Cys Ala Leu Pro Pro Arg Leu Lys Glu Met Lys Ser Gln Glu
                85                  90                  95

TCT GTG GCA GGT TCC AAA CTA GTG CTT CGG TGC GAG ACC AGT TCT GAA          343
Ser Val Ala Gly Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu
        100                 105                 110

TAC TCC TCT CTC AAG TTC AAG TGG TTC AAG AAT GGG AGT GAA TTA AGC          391
Tyr Ser Ser Leu Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser
      115                 120                 125

CGA AAG AAC AAA CCA GAA AAC ATC AAG ATA CAG AAA AGG CCG GGG AAG          439
Arg Lys Asn Lys Pro Glu Asn Ile Lys Ile Gln Lys Arg Pro Gly Lys
  130                 135                 140

TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT          487
Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr
145                 150                 155                 160

ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC          535
Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn
                165                 170                 175

ATC ACC ATT GTG GAG TCA AAC GGT AAG AGA TGC CTA CTG CGT GCT ATT          583
Ile Thr Ile Val Glu Ser Asn Gly Lys Arg Cys Leu Leu Arg Ala Ile
        180                 185                 190

TCT CAG TCT CTA AGA GGA GTG ATC AAG GTA TGT GGT CAC ACT                  625
Ser Gln Ser Leu Arg Gly Val Ile Lys Val Cys Gly His Thr
      195                 200                 205

TGAATCACGC AGGTGTGTGA AATCTCATTG TGAACAAATA AAAATCATGA AAGGAAAAAA        685

AAAAAAAAAA AATCGATGTC GACTCGAGAT GTGGCTGCAG GTCGACTCTA GAGGATCCC         744
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Figure 11 B

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CCTGCAG CAT CAA GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG      55
        His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu
        1               5                   10                  15

CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC GCC TTC CCC TCC TGC          103
Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser Cys
            20                  25                  30

GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAG          151
Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro Glu
        35                  40                  45

GCC AAC AGC AGC GGC GGG CCC GGC CGC CTT CCG AGC CTC CTT CCC CCC          199
Ala Lys Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro Pro
    50                  55                  60

TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT GTG          247
Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala Val
65                  70                  75                  80

CAA CGG TGC GCC TTG CCT CCC CGC TTG AAA GAG ATG AAG AGT CAG GAG          295
Gln Arg Cys Ala Leu Pro Pro Arg Leu Lys Glu Met Lys Ser Gln Glu
                85                  90                  95

TCT GTG GCA GGT TCC AAA CTA GTG CTT CGG TGC GAG ACC AGT TCT GAA          343
Ser Val Ala Gly Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu
            100                 105                 110

TAC TCC TCT CTC AAG TTC AAG TGG TTC AAG AAT GGG AGT GAA TTA AGC          391
Tyr Ser Ser Leu Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser
        115                 120                 125

CGA AAG AAC AAA CCA GAA AAC ATC AAG ATA CAG AAA AGG CCG GGG AAG          439
Arg Lys Asn Lys Gly Gly Asn Ile Lys Ile Gln Lys Arg Pro Gly Lys
    130                 135                 140

TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT          487
Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr
145                 150                 155                 160

ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC          535
Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn
                165                 170                 175
```

Figure 11 B'

```
ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA          583
Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly Thr
           180                 185                 190

AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT          631
Ser His Leu Val Lys Ser Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
       195                 200                 205

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC          679
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
   210                 215                 220

TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT          727
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn
225                 230                 235                 240

GTG CCC ATG AAA GTC CAA ACC CAA GAA AGT GCC CAA ATG AGT TTA CTG          775
Val Pro Met Lys Val Gln Thr Gln Glu Ser Ala Gln Met Ser Leu Leu
               245                 250                 255

GTG ATC GCT GCC AAA ACT ACG TAATGGCCAG CTTCTACAGT ACGTCCACTC             826
Val Ile Ala Ala Lys Thr Thr
           260

CCTTTCTGTC TCTGCCTGAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG TTGCCGCATC        886

TCCCCTCAGA TTCCTCCTAG AGCTAGATGC GTTTTACCAG GTCTAACATT GACTGCCTCT        946

GCCTGTCGCA TGAGAACATT AACACAAGCG ATTGTATGAC TTCCTCTGTC CGTGACTAGT       1006

GGGCTCTGAG CTACTCGTAG GTGCGTAAGG CTCCAGTGTT TCTGAAATTG ATCTTGAATT       1066

ACTGTGATAC GACATGATAG TCCCTCTCAC CCAGTGCAAT GACAATAAAG GCCTTGAAAA       1126

GTCAAAAAAA AAAAAAAAAA AAAAAATCGA TGTCGACTCG AGATGTGGCT GCAGGTCGAC       1186

TCTAGAG                                                                 1193
```

Figure 11 C

```
CCTGCAG CAT CAA GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG      55
        His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu
        1               5                   10                  15

CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC GCC TTC CCC TCC TGC          103
Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser Cys
            20                  25                  30

GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAG          151
Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro Glu
        35                  40                  45

GCC AAC AGC AGC GGC GGG CCC GGC CGC CTT CCG AGC CTC CTT CCC CCC          199
Ala Asn Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro Pro
    50                  55                  60

TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT GTG          247
Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala Val
65                  70                  75                  80

CAA CGG TGC GCC TTG CCT CCC CGC TTG AAA GAG ATG AAG AGT CAG GAG          295
Gln Arg Cys Ala Leu Pro Pro Arg Leu Lys Glu Met Lys Ser Gln Glu
                85                  90                  95

TCT GTG GCA GGT TCC AAA CTA GTG CTT CGG TGC GAG ACC AGT TCT GAA          343
Ser Val Ala Gly Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu
            100                 105                 110

TAC TCC TCT CTC AAG TTC AAG TGG TTC AAG AAT GGG AGT GAA TTA AGC          391
Tyr Ser Ser Leu Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser
        115                 120                 125

CGA AAG AAC AAA CCA GAA AAC ATC AAG ATA CAG AAA AGG CCG GGG AAG          439
Arg Lys Asn Lys Pro Glu Asn Ile Lys Ile Gln Lys Arg Pro Pro Lys
    130                 135                 140

TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT          487
Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr
145                 150                 155                 160
```

Figure 11 C'

```
ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC                535
Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn
                165                 170                 175

ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA                583
Ile Arg Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly Thr
            180                 185                 190

AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT                631
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
        195                 200                 205

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC                679
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
    210                 215                 220

TTG TGC AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC                727
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
225                 230                 235                 240

GTA ATG GCC AGC TTC TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT                775
Val Met Ala Ser Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro
                245                 250                 255

GAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG TTGCCGCATC TCCCCTCAGA TTCCGCCTAG          838
Glu

AGCTAGATGC GTTTTACCAG GTCTAACATT GACTGCCTCT GCCTGTCGCA TGAGAACATT              898

AACACAAGCG ATTGTATGAC TTCCTCTGTC CGTGACTAGT GGGCTCTGAG CTACTCGTAG              958

GTGCGTAAGG CTCCAGTGTT TCTGAAATTG ATCTTGAATT ACTGTGATAC GACATGATAG             1018

TCCCTCTCAC CCAGTGCAAT GACAATAAAG GCCTTGAAAA GTCAAAAAAA AAAAAAAAAA             1078

AAAAATCGAT GTCGACTCGA GATGTGGCTG                                              1108
```

Figure 12

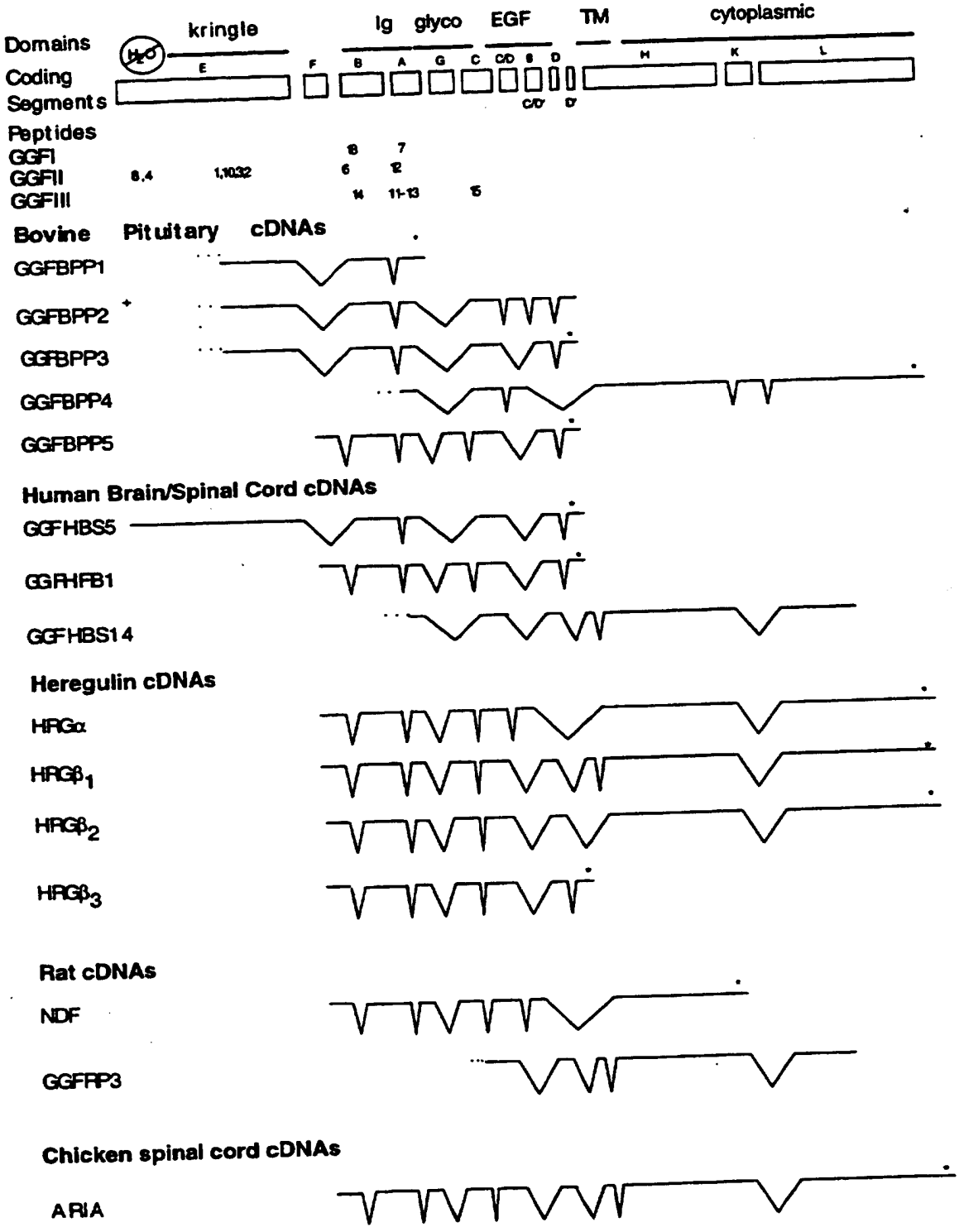

Figure 13 A

CODING SEGMENT F:

```
AGTTTCCCCC CCCAACTTGT CGGAACTCTG GGCTCGCGCG CAGGGCAGGA GCGGAGCGGC        60

GGCGGCTGCC CAGGCGATGC GAGCGCGGGC CGGACGGTAA TCGCCTCTCC CTCCTCGGGC       120

TGCGAGCGCG CCGGACCGAG GCAGCGACAG GAGCGGACCG CGGCGGGAAC CGAGGACTCC       180

CCAGCGGCGC GCCAGCAGGA GCCACCCCGC GAGNCGTGCG ACCGGGACGG AGCGCCCGCC       240

AGTCCCAGGT GGCCCGGACC GCACGTTGCG TCCCCGCGCT CCCCGCCGGC GACAGGAGAC       300

GCTCCCCCCC ACGCCGCGCG CGCCTCGGCC CGGTCGCTGG CCCGCCTCCA CTCCGGGGAC       360
                |||| | |||||| ||  ||| |||| | | | | ||  |||  |||||
                CGCGAG CGCCTCAGCG CGGCCGCTCG CTCTC..CCC CTCGAGGGAC

AAACTTTTCC CGAAGCCGAT CCCAGCCCTC GGACCCAAAC TTGTCGCGCG TCGCCTTCGC       420
|||||||||| | || ||||| || ||||||  ||||| ||              ||||||  |||
AAACTTTTCC CAAACCCGAT CCGAGCCCTT GGACCAAA.. .........C TCGCCTGCGC

                                                  Met Ser Glu Arg Arg
CGGGAGCCGT CCGCGCAGAG CGTGCACTTC TCGGGCGAG ATG TCG GAG CGC AGA          474
|| |||||||| ||||| |||| ||  | | || || |||||| ||| ||  ||| ||| | |
CGAGAGCCGT CCGCGTAGAG CGCTC.CGTC TCCGGCGAG ATG TCC GAG CGC AAA
                                                        K

Glu Gly Lys Gly Lys Gly Lys Gly Gly Lys Lys Asp Arg Gly Ser Gly
GAA GGC AAA GGC AAG GGG AAG GGC GGC AAG AAG GAC CGA GGC TCC GGG          522
||| ||| | | ||| ||  ||| ||| |||     ||| ||| ||  ||| ||| ||| ||
GAA GGC AGA GGC AAA GGG AAG GGC AAG AAG AAG GAG CGA GGC TCC GGC
        R                       K           E

Lys Lys Pro Val Pro Ala Ala Gly Gly Pro Ser Pro Ala
AAG AAG CCC GTG CCC GCG GCT GGC GGC CCG AGC CCA G                       559
||| ||| ||  | |  || ||| ||  |||  || | | ||| ||| |
AAG AAG CCG GAG TCC GCG GCG GGC AGC CAG AGC CCA G
          E   S
```

Figure 13 B

CODING SEGMENT E:

```
CC CAT CAN GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG          47
   His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser
     1               5                  10                  15

CTG CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC GCC TTC CCC TCC          95
Leu Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser
                20                  25                  30

TGC GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC         143
Cys Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro
            35                  40                  45

GAG GCC AAC AGC AGC GGC GGG CCC GGC CGC CTT CCG AGC CTC CTT CCC         191
Glu Ala Asn Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro
        50                  55                  60

CCC TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT         239
Pro Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala
    65                  70                  75

                                                                        252
GTG CAA CGG TGC G
Val Gln Arg Cys
 80
```

Figure 13 C

CODING SEGMENT B:

```
Leu Pro Pro Arg Leu Lys Glu His Lys Ser Gln Glu Ser Val Ala Gly          48
CCT TGC CTC CCC GCT TGA AAG AGA TGA AGA GTC AGG AGT CTG TGG CAG
||| ||| ||| ||| | | ||| ||| ||| ||| | | ||| | | |   | |||
CCT TGC CTC CCC GAT TGA AAG AGA TGA AAA GCC AGG AAT CGG CTG CAG
              Q                                   A

Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser Ser Leu          96
GTT CCA AAC TAG TGC TTC GGT GCG AGA CCA GTT CTG AAT ACT CCT CTC
||| ||| ||| ||| | | ||| ||| | | | ||| ||| ||| ||| ||| ||| |||
GTT CCA AAC TAG TCC TTC GGT GTG AAA CCA GTT CTG AAT ACT CCT CTC

Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser Arg Lys Asn Lys         144
TCA AGT TCA AGT GGT TCA AGA ATG GGA GTG AAT TAA GCC GAA AGA ACA
|||   | ||| ||| ||| ||| ||| ||| ||| || ||| | |   | ||| | | |||
TCA GAT TCA AGT GGT TCA AGA ATG GGA ATG AAT TGA ATC GAA AAA ACA
R                           N           N

Pro Gly Asn Ile Lys Ile Gln Lys Arg Pro Gly                             178
AAC CAC AAA ACA TCA AGA TAC AGA AAA GGC CGG G
||| ||| ||| | ||| ||| ||| | | ||| || | | |
AAC CAC AAA ATA TCA AGA TAC AAA AAA AGC CAG G
                        K
```

Figure 13 D

CODING SEGMENT A:

```
  Lys Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly
G AAG TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA          46
  ||| ||| ||| ||| ||| ||| | | ||| ||  ||| ||| ||| ||| ||| |||
G AAG TCA GAA CTT CGC ATT AAC AAA GCA TCA CTG GCT GAT TCT GGA
                        N

Glu Tyr Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser
GAA TAT ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT          94
||  ||| ||| ||| ||| ||| ||| ||| ||| ||  ||| ||| ||| ||| ||| |||
GAG TAT ATG TGC AAA GTG ATC AGC AAA TTA GGA AAT GAC AGT GCC TCT

Ala Asn Ile Thr Ile Val Glu Ser Asn Ala
GCC AAC ATC ACC ATT GTG GAG TCA AAC G                                 122
||| ||  ||| ||| ||  ||| ||  ||| ||| |
GCC AAT ATC ACC ATC GTG GAA TCA AAC G
```

Figure 13 E

CODING SEGMENT A':

```
TCTAAAACTA CAGAGACTGT ATTTTCATGA TCATCATAGT TCTGTGAAAT ATACTTAAAC      60

CGCTTTGGTC CTGATCTTGT AGG AAG TCA GAA CTT CGC ATT AGC AAA GCG          110
                          Lys Ser Glu Leu Arg Ile Ser Lys Ala
                            1               5

TCA CTG GCT GAT TCT GGA GAA TAT ATG TGC AAA GTG ATC AGC AAA CTA        158
Ser Leu Ala Asp Ser Gly Glu Tyr Met Cys Lys Val Ile Ser Lys Leu
 10                  15                  20                  25

GGA AAT GAC AGT GCC TCT GCC AAC ATC ACC ATT GTG GAG TCA AAC GGT        206
Gly Asn Asp Ser Ala Ser Ala Asn Ile Thr Ile Val Glu Ser Asn Gly
                30                  35                  40

AAG AGA TGC CTA CTG CGT GCT ATT TCT CAG TCT CTA AGA GGA GTG ATC        254
Lys Arg Cys Leu Leu Arg Ala Ile Ser Gln Ser Leu Arg Gly Val Ile
            45                  50                  55

AAG GTA TGT GGT CAC ACT TGAATCACGC AGGTGTGTGA AATCTCATTG               302
Lys Val Cys Gly His Thr
        60

TGAACAAATA AAAATCATGA AAGGAAAACT CTATGTTTGA AATATCTTAT GGGTCCTCCT      362

GTAAAGCTCT TCACTCCATA AGGTGAAATA GACCTGAAAT ATATATAGAT TATTT           417
```

Figure 13 F

CODING SEGMENT G:

```
Glu Ile Thr Thr Gly Met Pro Ala Ser Thr Glu Thr Ala Tyr Val Ser
 AG ATC ACC ACT GGC ATG CCA GCC TCA ACT GAG ACA GCG TAT GTG TCT      47
 || ||| | | ||| ||  ||| ||| ||| ||| ||  |  ||  ||| ||| ||| |||
 AG ATC ATC ACT GGT ATG CCA GCC TCA ACT GAA GGA GCA TAT GTG TCT
             I                           G

Ser Glu Ser Pro Ile Arg Ile Ser Val Ser Thr Glu Gly Thr Asn Thr
TCA GAG TCT CCC ATT AGA ATA TCA GTA TCA ACA GAA GGA ACA AAT ACT      95
||| ||| ||| ||| ||| ||| ||| ||| ||| ||  ||| ||| ||  ||| ||| |||
TCA GAG TCT CCC ATT AGA ATA TCA GTA TCC ACA GAA GGA GCA AAT ACT
                                                  A

Ser Ser Ser
TCT TCA T                                                          102
||| ||| |
TCT TCA T
```

Figure 13 G

CODING SEGMENT C:

```
   Thr Ser Thr Ser Thr Ala Gly Thr Ser His Leu Val Lys Cys Ala
CC ACA TCC ACA TCT ACA GCT GGG ACA AGC CAT CTT GTC AAG TGT GCA      47
|  ||| ||  ||| ||  ||  ||  ||| ||| ||| ||| ||| ||  ||  ||| ||
CT ACA TCT ACA TCC ACC ACT GGG ACA AGC CAT CTT GTA AAA TGT GCG
                       T

Glu Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys Phe Met Val
GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGC GAG TGC TTC ATG GTG      95
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||  ||| ||| ||| ||| |||
GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGG GAG TGC TTC ATG GTG

Lys Asp Leu Ser Asn Pro Ser Arg Tyr Leu Cys
AAA GAC CTT TCA AAT CCC TCA AGA TAC TTG TGC                         128
||| ||| ||| ||| ||  ||| ||  ||| ||| ||| |||
AAA GAC CTT TCA AAC CCC TCG AGA TAC TTG TGC
```

Figure 13 H

CODING SEGMENT C/D:

```
Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn Val Pro
AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT GTG CCC        48
||| ||| ||| ||| ||| ||| ||| ||| ||  ||| ||| ||| ||| ||| ||| |||
AAG TGC CAA CCT GGA TTC ACT GGA GCA AGA TGT ACT GAG AAT GTG CCC

Met Lys Val Gln Thr Gln Glu
ATG AAA GTC CAA ACC CAA GAA                                          69
||| ||| ||| ||| | | ||| |||
ATG AAA GTC CAA AAC CAA GAA
              N
```

Figure 13 I

CODING SEGMENT C/D':

```
Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met
AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG      48
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG

Ala Ser Phe Tyr
GCC AGC TTC TAC                                                      60
||| ||| ||| |||
GCC AGC TTC TAC
```

Figure 13 J

CODING SEGMENT D:

```
Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro Glu  *
AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA TAG
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA TAG
```

36

Figure 13 K

CODING SEGMENT D':

```
Lys His Leu Gly Ile Glu Phe Met Glu
AAG CAT CTT GGG ATT GAA TTT ATG GAG
```

27

Figure 13 L

CODING SEGMENT H:

```
Lys Ala Glu Glu Leu Tyr Gln Lys Arg Val Leu Thr Ile Thr Gly Ile
AAA GCG GAG GAG CTC TAC CAG AAG AGA GTG CTC ACC ATT ACC GGC ATT        48
||| ||| ||| ||| ||  ||| ||| ||| ||| ||| ||  ||| ||  ||| ||| ||
AAA GCG GAG GAG CTG TAC CAG AAG AGA GTG CTG ACC ATA ACC GGC ATC

Cys Ile Ala Leu Leu Val Val Gly Ile Met Cys Val Val Val Tyr Cys
TGC ATC GCG CTG CTC GTG GTT GGC ATC ATG TGT GTG GTG GTC TAC TGC        96
||| ||| ||  ||  ||  ||| ||  ||| ||| ||| ||| ||| ||| | |  ||| |||
TGC ATC GCC CTC CTT GTG GTC GGC ATC ATG TGT GTG GTG GCC TAC TGC
                                           A

Lys Thr Lys Lys Gln Arg Lys Lys Leu His Asp Arg Leu Arg Gln Ser
AAA ACC AAG AAA CAA CGG AAA AAG CTT CAT GAC CGG CTT CGG CAG AGC       144
||| ||| ||| ||| ||  ||| ||| ||| ||  ||| ||| ||  ||| ||| ||| |||
AAA ACC AAG AAA CAG CGG AAA AAG CTG CAT GAC CGT CTT CGG CAG AGC

Leu Arg Ser Glu Arg Asn Thr Met Met Asn Val Ala Asn Gly Pro His
CTT CGG TCT GAA AGA AAC ACC ATG ATG AAC GTA GCC AAC GGG CCC CAC       192
||| ||| ||| ||| ||  ||| |   ||| ||| |||  |  ||| ||  ||| ||  |||
CTT CGG TCT GAA CGA AAC AAT ATG ATG AAC ATT GCC AAT GGG CCT CAC
                        N                I

His Pro Asn Pro Pro Pro Glu Asn Val Gln Leu Val Asn Gln Tyr Val
CAC CCC AAT CCG CCC CCC GAG AAC GTG CAG CTG GTG AAT CAA TAC GTA       240
||  ||  ||  ||  ||| ||| ||| ||  ||  ||| ||| ||| ||| ||| ||| |||
CAT CCT AAC CCA CCC CCC GAG AAT GTC CAG CTG GTG AAT CAA TAC GTA

Ser Lys Asn Val Ile Ser Ser Glu His Ile Val Glu Arg Glu Ala Glu
TCT AAA AAT GTC ATC TCT AGC GAG CAT ATT GTT GAG AGA GAG GCG GAG       288
||| ||| ||  ||| ||| ||  ||  ||| ||| ||| ||| ||| ||| ||  ||  |||
TCT AAA AAC GTC ATC TCC AGT GAG CAT ATT GTT GAG AGA GAA GCA GAG
```

Figure 13 L'

```
Ser Ser Phe Ser Thr Ser His Tyr Thr Ser Thr Ala His His Ser Thr
AGC TCT TTT TCC ACC AGT CAC TAC ACT TCG ACA GCT CAT CAT TCC ACT      336
|   ||  ||| ||| ||| ||| ||| ||  ||| ||  ||| ||  ||| ||| |||
ACA TCC TTT TCC ACC AGT CAC TAT ACT TCC ACA GCC CAT CAC TCC ACT
T

Thr Val Thr Gln Thr Pro Ser His Ser Trp Ser Asn Gly His Thr Glu
ACT GTC ACT CAG ACT CCC AGT CAC AGC TGG AGC AAT GGA CAC ACT GAA      384
||| ||| ||  ||| ||| ||  ||  ||| ||| ||| ||| ||  ||| ||| ||| |||
ACT GTC ACC CAG ACT CCT AGC CAC AGC TGG AGC AAC GGA CAC ACT GAA

Ser Ile Ile Ser Glu Ser His Ser Val Ile Val Met Ser Ser Val Glu
AGC ATC ATT TCG GAA AGC CAC TCT GTC ATC GTG ATG TCA TCC GTA GAA      432
|||  ||| ||  ||  ||| ||| ||| ||| ||  ||| ||| ||| ||| ||| ||| |||
AGC ATC CTT TCC GAA AGC CAC TCT GTA ATC GTG ATG TCA TCC GTA GAA
         L

Asn Ser Arg His Ser Ser Pro Thr Gly Gly Pro Arg Gly Arg Leu Asn
AAC AGT AGG CAC AGC AGC CCG ACT GGG GGC CCG AGA GGA CGT CTC AAT      480
|||  ||| ||| ||| ||| ||| ||  ||| ||| ||| ||  ||| ||| ||  ||| |||
AAC AGT AGG CAC AGC AGC CCA ACT GGG GGC CCA AGA GGA CGT CTT AAT

Gly Leu Gly Gly Pro Arg Glu Cys Asn Ser Phe Leu Arg His Ala Arg
GGC TTG GGA GGC CCT CGT GAA TGT AAC AGC TTC CTC AGG CAT GCC AGA      528
|||     |||  ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
GGC ACA GGA GGC CCT CGT GAA TGT AAC AGC TTC CTC AGG CAT GCC AGA
    T

Glu Thr Pro Asp Ser Tyr Arg Asp Ser Pro His Ser Glu Arg
GAA ACC CCT GAC TCC TAC CGA GAC TCT CCT CAT AGT GAA AG              569
|||  ||| |||  ||  ||| ||| ||| ||| ||| ||| ||| ||| ||| ||
GAA ACC CCT GAT TCC TAC CGA GAC TCT CCT CAT AGT GAA AG
```

Figure 13 M

CODING SEGMENT K:

```
A CAT AAC CTT ATA GCT GAG CTA AGG AGA AAC AAG GCC CAC AGA TCC          46
  His Asn Leu Ile Ala Glu Leu Arg Arg Asn Lys Ala His Arg Ser
    1               5                  10                  15

AAA TGC ATG CAG ATC CAG CTT TCC GCA ACT CAT CTT AGA GCT TCT TCC        94
Lys Cys Met Gln Ile Gln Leu Ser Ala Thr His Leu Arg Ala Ser Ser
                20                  25                  30

ATT CCC CAT TGG GCT TCA TTC TCT AAG ACC CCT TGG CCT TTA GGA AG        141
Ile Pro His Trp Ala Ser Phe Ser Lys Thr Pro Trp Pro Leu Gly Arg
            35                  40                  45
```

Figure 13 N

CODING SEGMENT L:

```
  Tyr Val Ser Ala Met Thr Thr Pro Ala Arg Met Ser Pro Val Asp
G TAT GTA TCA GCA ATG ACC ACC CCG GCT CGT ATG TCA CCT GTA GAT          46
| ||| ||  ||| ||  ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
G TAT GTG TCA GCC ATG ACC ACC CCG GCT CGT ATG TCA CCT GTA GAT

Phe His Thr Pro Ser Ser Pro Lys Ser Pro Pro Ser Glu Met Ser Pro
TTC CAC ACG CCA AGC TCC CCC AAG TCA CCC CCT TCG GAA ATG TCC CCG          94
||| ||| ||| ||| ||| ||| ||| ||  ||  ||| ||| ||| ||| ||| ||| ||
TTC CAC ACG CCA AGC TCC CCC AAA TCG CCC CCT TCG GAA ATG TCT CCA

Pro Val Ser Ser Thr Thr Val Ser Met Pro Ser Met Ala Val Ser Pro
CCC GTG TCC AGC ACG ACG GTC TCC ATG CCC TCC ATG GCG GTC AGT CCC         142
||| ||| ||| ||| | | ||| ||  ||| ||| ||  ||| ||| ||| ||| ||  |||
CCC GTG TCC AGC ATG ACG GTG TCC ATG CCT TCC ATG GCG GTC AGC CCC
                M

Phe Val Glu Glu Glu Arg Pro Leu Leu Leu Val Thr Pro Pro Arg Leu
TTC GTG GAA GAG GAG AGA CCC CTG CTC CTT GTG ACG CCA CCA CGG CTG         190
|||  ||  ||| ||  ||| ||| ||  ||  ||  ||| ||  ||| |||  ||  |||
TTC ATG GAA GAA GAG AGA CCT CTA CTT CTC GTG ACA CCA CCA AGG CTG
    N

Arg Glu Lys  -  Tyr Asp His His Ala Gln Gln Phe Asn Ser Phe His
CGG GAG AAG ... TAT GAC CAC CAC GCC CAG CAA TTC AAC TCG TTC CAC         238
|||  ||  |||     | |  ||| ||  |||  |  |||  ||  |||  |  ||  |||  |||
CGG GAG AAG AAG TTT GAC CAT CAC CCT CAG CAG TTC AGC TCC TTC CAC
          K   F               P

Cys Asn Pro Ala His Glu Ser Asn Ser Leu Pro Pro Ser Pro Leu Arg
TGC AAC CCC GCG CAT GAG AGC AAC AGC CTG CCC CCC AGC CCC TTG AGG         286
  |  |||  |||  |||  |||  ||  ||  |||  |||  ||  ||  |  |||  |||  |||  |||
CAC AAC CCC GCG CAT GAC AGT AAC AGC CTC CCT GCT AGC CCC TTG AGG
N                   D                   A
```

Figure 13 N'

```
Ile Val Glu Asp Glu Glu Tyr Glu Thr Thr Gln Glu Tyr Glu Pro Ala
ATA GTG GAG GAT GAG GAA TAT GAA ACG ACC CAG GAG TAC GAA CCA GCT      334
||| ||| ||| ||| ||| ||  ||| ||| ||| ||| ||  ||| ||| ||  ||| ||
ATA GTG GAG GAT GAG GAG TAT GAA ACG ACC CAA GAG TAC GAG CCA GCC

Gln Glu Pro Val Lys Lys Leu Thr Asn Ser Ser Arg Arg Ala Lys Arg
CAA GAG CCG GTT AAG AAA CTC ACC AAC AGC AGC CGG CGG GCC AAA AGA      382
||| ||| ||  ||| ||| ||| |||  || |||     ||| ||| ||| ||| ||| |||
CAA GAG CCT GTT AAG AAA CTC GCC AA. ..T AGC CGG CGG GCC AAA AGA
                           A

Thr Lys Pro Asn Gly His Ile Ala His Arg Leu Glu Met Asp Asn Asn
ACC AAG CCC AAT GGT CAC ATT GCC CAC AGG TTG GAA ATG GAC AAC AAC      430
||| ||| ||| ||| ||  ||| ||| ||   || ||  ||| ||| ||| ||| | | |||
ACC AAG CCC AAT GGC CAC ATT GCT AAC AGA TTG GAA GTG GAC AGC AAC
                               N           V       S

Thr Gly Ala Asp Ser Ser Asn Ser Glu Ser Glu Thr Glu Asp Glu Arg
ACA GGC GCT GAC AGC AGT AAC TCA GAG AGC GAA ACA GAG GAT GAA AGA      478
|||  ||  |   |  ||| ||| ||| ||| ||| ||  ||| ||| ||  ||| ||| |||
ACA AGC TCC CAG AGC AGT AAC TCA GAG AGT GAA ACA GAA GAT GAA AGA
    S   S   Q
```

Figure 13 N''

```
Val Gly Glu Asp Thr Pro Phe Leu Ala Ile Gln Asn Pro Leu Ala Ala
GTA GGA GAA GAT ACG CCT TTC CTG GCC ATA CAG AAC CCC CTG GCA GCC
||| ||  ||| ||| ||| ||| ||| ||| | | ||| ||| ||| ||| ||| ||| |||
GTA GGT GAA GAT ACG CCT TTC CTG GGC ATA CAG AAC CCC CTG GCA GCC
                                 G                                526

Ser Leu Glu Ala Ala Pro Ala Phe Arg Leu Val Asp Ser Arg Thr Asn
AGT CTC GAG GCG GCC CCT GCC TTC CGC CTG GTC GAC AGC AGG ACT AAC
|||  ||  ||| ||   |  ||| ||| ||| ||| ||| |  ||| ||| ||| ||| |||
AGT CTT GAG GCA ACA CCT GCC TTC CGC CTG GCT GAC AGC AGG ACT AAC
                T                       A                        574

Pro Thr Gly Gly Phe Ser Pro Gln Glu Glu Leu Gln Ala Arg Leu Ser
CCA ACA GGC GGC TTC TCT CCG CAG GAA GAA TTG CAG GCC AGG CTC TCC
|||  ||  ||| ||  ||| ||   |  ||| ||| ||| |  ||| ||| ||| ||  ||
CCA GCA GGC CGC TTC TCG ACA CAG GAA GAA ATC CAG GCC AGG CTG TCT
    A       R           T                 I                      622

Gly Val Ile Ala Asn Gln Asp Pro Ile Ala Val  *
GGT GTA ATC GCT AAC CAA GAC CCT ATC GCT GTC TAA AAC CGA AAT ACA
 ||  ||| ||  ||| ||| ||| ||| ||| ||  ||| ||  ||| ||| | | ||| | |
AGT GTA ATT GCT AAC CAA GAC CCT ATT GCT GTA TAA AAC CTA AAT AAA
S                                                                672

CCC ATA GAT TCA CCT GTA AAA CTT TAT TTT ATA TAA TAA AGT ATT CCA
| | ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
CAC ATA GAT TCA CCT GTA AAA CTT TAT TTT ATA TAA TAA AGT ATT CCA
                                                                 718

CCT TAA ATT AAA CAA
||| ||| ||| ||| |||
CCT TAA ATT AAA CAA                                              733
```

Figure 13 O

HUMAN CODING SEGMENT E:

```
ATG AGA TGG CGA CGC GCC CCG CGC CGC TCC GGG CGT CCC GGC CCC CGG          48
Met Arg Trp Arg Arg Ala Pro Arg Arg Ser Gly Arg Pro Gly Pro Arg
1               5                  10                  15

GCC CAG CGC CCC GGC TCC GCC GCC CGC TCG TCG CCG CCG CTG CCG CTG          96
Ala Gln Arg Pro Gly Ser Ala Ala Arg Ser Ser Pro Pro Leu Pro Leu
            20                  25                  30

CTG CCA CTA CTG CTG CTG CTG GGG ACC GCG GCC CTG GCG CCG GGG GCG         144
Leu Pro Leu Leu Leu Leu Leu Gly Thr Ala Ala Leu Ala Pro Gly Ala
        35                  40                  45

GCG GCC GGC AAC GAG GCG GCT CCC GCG GGG GCC TCG GTG TGC TAC TCG         192
Ala Ala Gly Asn Glu Ala Ala Pro Ala Gly Ala Ser Val Cys Tyr Ser
    50                  55                  60

TCC CCG CCC AGC GTG GGA TCG GTG CAG GAG CTA GCT CAG CGC GCC GCG         240
Ser Pro Pro Ser Val Gly Ser Val Gln Glu Leu Ala Gln Arg Ala Ala
65                  70                  75                  80

GTG GTG ATC GAG GGA AAG GTG CAC CCG CAG CGG CGG CAG CAG GGG GCA         288
Val Val Ile Glu Gly Lys Val His Pro Gln Arg Arg Gln Gln Gly Ala
                85                  90                  95

CTC GAC AGG AAG GCG GCG GCG GCG GCG GGC GAG GCA GGG GCG TGG GGC         336
Leu Asp Arg Lys Ala Ala Ala Ala Ala Gly Glu Ala Gly Ala Trp Gly
            100                 105                 110

GGC GAT CGC GAG CCG CCA GCC GCG GGC CCA CGG GCG CTG GGG CCG CCC         384
Gly Asp Arg Glu Pro Pro Ala Ala Gly Pro Arg Ala Leu Gly Pro Pro
        115                 120                 125

GCC GAG GAG CCG CTG CTC GCC GCC AAC GGG ACC GTG CCC TCT TGG CCC         432
Ala Glu Glu Pro Leu Leu Ala Ala Asn Gly Thr Val Pro Ser Trp Pro
    130                 135                 140

ACC GCC CCG GTG CCC AGC GCC GGC GAG CCC GGG GAG GAG GCG CCC TAT         480
Thr Ala Pro Val Pro Ser Ala Gly Glu Pro Gly Glu Glu Ala Pro Tyr
145                 150                 155                 160

CTG GTG AAG GTG CAC CAG GTG TGG GCG GTG AAA GCC GGG GGC TTG AAG         528
Leu Val Lys Val His Gln Val Trp Ala Val Lys Ala Gly Gly Leu Lys
                165                 170                 175

AAG GAC TCG CTG CTC ACC GTG CGC CTG GGG ACC TGG GGC CAC CCC GCC         576
Lys Asp Ser Leu Leu Thr Val Arg Leu Gly Thr Trp Gly His Pro Ala
            180                 185                 190

TTC CCC TCC TGC GGG AGG CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC         624
Phe Pro Ser Cys Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe
        195                 200                 205

ATG GAG CCC GAC GCC AAC AGC ACC AGC CGC GCG CCG GCC GCC TTC CGA         672
Met Glu Pro Asp Ala Asn Ser Thr Ser Arg Ala Pro Ala Ala Phe Arg
    210                 215                 220

GCC TCT TTC CCC CCT CTG GAG ACG GGC CGG AAC CTC AAG AAG GAG GTC         720
Ala Ser Phe Pro Pro Leu Glu Thr Gly Arg Asn Leu Lys Lys Glu Val
225                 230                 235                 240

AGC CGG GTG CTG TGC AAG CGG TGC G                                       745
Ser Arg Val Leu Cys Lys Arg Cys
                245
```

Figure 14 A

```
AGTTTCCCCC CCCAACTTGT CGGAACTCTG GGCTCGCGCG CAGGGCAGGA GCGGAGCGGC      60

GGCGGCTGCC CAGGCGATGC GAGCGCGGGC CGGACGGTAA TCGCCTCTCC CTCCTCGGGC     120

TGCGAGCGCG CCGGACCGAG GCAGCGACAG GAGCGGACCG CGGCGGGAAC CGAGGACTCC     180

CCAGCGGCGC GCCAGCAGGA GCCACCCCGC GAGCGTGCGA CCGGGACGGA GCGCCCGCCA     240

GTCCCAGGTG GCCCGGACCG CACGTTGCGT CCCCGCGCTC CCCGCCGGCG ACAGGAGACG     300

CTCCCCCCCA CGCCGCGCGC GCCTCGGCCC GGTCGCTGGC CCGCCTCCAC TCCGGGGACA     360

AACTTTTCCC GAAGCCGATC CCAGCCCTCG GACCCAAACT TGTCGCGCGT CGCCTTCGCC     420

GGGAGCCGTC CGCGCAGAGC GTGCACTTCT CGGGCGAG ATG TCG GAG CGC AGA          475
                                          Met Ser Glu Arg Arg
                                           1               5

GAA GGC AAA GGC AAG GGG AAG GGC GGC AAG AAG GAC CGA GGC TCC GGG        523
Glu Gly Lys Gly Lys Gly Lys Gly Gly Lys Lys Asp Arg Gly Ser Gly
                10                  15                  20

AAG AAG CCC GTG CCC GCG GCT GGC GGC CCG AGC CCA GCC TTG CCT CCC       571
Lys Lys Pro Val Pro Ala Ala Gly Gly Pro Ser Pro Ala Leu Pro Pro
            25                  30                  35

CGC TTG AAA GAG ATG AAG ATG CAG GAG TCT GTG GCA GGT TCC AAA CTA       619
Arg Leu Lys Glu Met Lys Ser Gln Glu Ser Val Ala Gly Ser Lys Leu
        40                  45                  50

GTG CTT CGG TGC GAG ACC AGT TCT GAA TAC TCC TCT CTC AAG TTC AAG       667
Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser Ser Leu Lys Phe Lys
    55                  60                  65

TGG TTC AAG AAT GGG AGT GAA TTA AGC CGA AAG AAC AAA CCA CAA AAC       715
Trp Phe Lys Asn Gly Ser Glu Leu Ser Arg Lys Asn Lys Pro Gln Asn
70                  75                  80                  85

ATC AAG ATA CAG AAA AGG CCG GGG AAG TCA GAA CTT CGC ATT AGC AAA       763
Ile Lys Ile Gln Lys Arg Pro Gly Lys Ser Glu Leu Arg Ile Ser Lys
                90                  95                  100

GCG TCA CTG GCT GAT TCT GGA GAA TAT ATG TGC AAA GTG ATC AGC AAA       811
Ala Ser Leu Ala Asp Ser Gly Glu Tyr Met Cys Lys Val Ile Ser Lys
            105                 110                 115
```

Figure 14 B

```
CTA GGA AAT GAC AGT GCC TCT GCC AAC ATC ACC ATT GTG GAG TCA AAC        859
Leu Gly Asn Asp Ser Ala Ser Ala Asn Ile Thr Ile Val Glu Ser Asn
        120                 125                 130

GAG ATC ACC ACT GGC ATG CCA GCC TCA ACT GAG ACA GCG TAT GTG TCT        907
Glu Ile Thr Thr Gly Met Pro Ala Ser Thr Glu Thr Ala Tyr Val Ser
    135                 140                 145

TCA GAG TCT CCC ATT AGA ATA TCA GTA TCA ACA GAA GGA ACA AAT ACT        955
Ser Glu Ser Pro Ile Arg Ile Ser Val Ser Thr Glu Gly Thr Asn Thr
150                 155                 160                 165

TCT TCA TCC ACA TCC ACA TCT ACA GCT GGG ACA AGC CAT CTT GTC AAG       1003
Ser Ser Ser Thr Ser Thr Ser Thr Ala Gly Thr Ser His Leu Val Lys
                170                 175                 180

TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGC GAG TGC TTC       1051
Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys Phe
            185                 190                 195

ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC TTG TGC AAG TGC CCA       1099
Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr Leu Cys Lys Cys Pro
        200                 205                 210

AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC       1147
Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe
    215                 220                 225

TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA TAGGCGCATG            1193
Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro Glu
230                 235                 240

CTCAGTCGGT GCCGCTTTCT TGTTGCCGCA TCTCCCCTCA GATTCAACCT AGAGCTAGAT     1253

GCGTTTTACC AGGTCTAACA TTGACTGCCT CTGCCTGTCG CATGAGAACA TTAACACAAG     1313

CGATTGTATG ACTTCCTCTG TCCGTGACTA GTGGGCTCTG AGCTACTCGT AGGTGCGTAA     1373

GGCTCCAGTG TTTCTGAAAT TGATCTTGAA TTACTGTGAT ACGACATGAT AGTCCCTCTC     1433

ACCCAGTGCA ATGACAATAA AGGCCTTGAA AAGTCTCACT TTTATTGAGA AAATAAAAAT     1493

CGTTCCACGG GACAGTCCCT CTTCTTTATA AAATGACCCT ATCCTTGAAA AGGAGGTGTG     1553

TTAAGTTGTA ACCAGTACAC ACTTGAAATG ATGGTAAGTT CGCTTCGGTT CAGAATGTGT     1613

TCTTTCTGAC AAATAAACAG AATAAAAAAA AAAAAAAAAA A                         1654
```

Figure 15 A

```
CAT CAN GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG          48
His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu
1               5                   10                  15

CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC GCC TTC CCC TCC TGC          96
Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser Cys
            20                  25                  30

GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAG         144
Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro Glu
        35                  40                  45

GCC AAC AGC AGC GGC GGG CCC GGC CGC CTT CCG AGC CTC CTT CCC CCC         192
Ala Asn Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro Pro
    50                  55                  60

TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT GTG         240
Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala Val
65                  70                  75                  80

CAA CGG TGC GCC TTG CCT CCC CGC TTG AAA GAG ATG AAG AGT CAG GAG         288
Gln Arg Cys Ala Leu Pro Pro Arg Leu Lys Glu Met Lys Ser Gln Glu
                85                  90                  95

TCT GTG GCA GGT TCC AAA CTA GTG CTT CGG TGC GAG ACC AGT TCT GAA         336
Ser Val Ala Gly Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu
            100                 105                 110

TAC TCC TCT CTC AAG TTC AAG TGG TTC AAG AAT GGG AGT GAA TTA AGC         384
Tyr Ser Ser Leu Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser
        115                 120                 125

CGA AAG AAC AAA CCA GAA AAC ATC AAG ATA CAG AAA AGG CCG GGG AAG         432
Arg Lys Asn Lys Pro Glu Asn Ile Lys Ile Gln Lys Arg Pro Gly Lys
    130                 135                 140

TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT         480
Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr
145                 150                 155                 160

ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC         528
Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn
                165                 170                 175
```

Figure 15 B

```
ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA                576
Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly Thr
            180                 185                 190

AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT                624
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
        195                 200                 205

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC                672
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
    210                 215                 220

TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT                720
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn
225                 230                 235                 240

GTG CCC ATG AAA GTC CAA ACC CAA GAA AAG TGC CCA AAT GAG TTT ACT                768
Val Pro Met Lys Val Gln Thr Gln Glu Lys Cys Pro Asn Glu Phe Thr
                245                 250                 255

GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC TAC AGT ACG TCC                816
Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe Tyr Ser Thr Ser
            260                 265                 270

ACT CCC TTT CTG TCT CTG CCT GAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG               870
Thr Pro Phe Leu Ser Leu Pro Glu
        275                 280

TTGCCGCATC TCCCCTCAGA TTCCNCCTAG AGCTAGATGC GTTTTACCAG GTCTAACATT              930

GACTGCCTCT GCCTGTCGCA TGAGAACATT AACACAAGCG ATTGTATGAC TTCCTCTGTC              990

CGTGACTAGT GGGCTCTGAG CTACTCGTAG GTGCGTAAGG CTCCAGTGTT TCTGAAATTG             1050

ATCTTGAATT ACTGTGATAC GACATGATAG TCCCTCTCAC CCAGTGCAAT GACAATAAAG             1110

GCCTTGAAAA GTCAAAAAAA AAAAAAAAAA                                              1140
```

Figure 16 A

```
G AAG TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA          49
  Lys Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu
   1               5                  10                  15

TAT ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC          97
Tyr Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala
            20                  25                  30

AAC ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG         145
Asn Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly
        35                  40                  45

ACA AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG         193
Thr Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val
    50                  55                  60

AAT GGA GGC GAC TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA         241
Asn Gly Gly Asp Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg
65                  70                  75                  80

TAC TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG         289
Tyr Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu
                85                  90                  95

AAT GTG CCC ATG AAA GTC CAA ACC CAA GAA AAA GCG GAG GAG CTC TAC         337
Asn Val Pro Met Lys Val Gln Thr Gln Glu Lys Ala Glu Glu Leu Tyr
            100                 105                 110

CAG AAG AGA GTG CTC ACC ATT ACC GGC ATT TGC ATC GCG CTG CTC GTG         385
Gln Lys Arg Val Leu Thr Ile Thr Gly Ile Cys Ile Ala Leu Leu Val
        115                 120                 125

GTT GGC ATC ATG TGT GTG GTG GTC TAC TGC AAA ACC AAG AAA CAA CGG         433
Val Gly Ile Met Cys Val Val Val Tyr Cys Lys Thr Lys Lys Gln Arg
    130                 135                 140

AAA AAG CTT CAT GAC CGG CTT CGG CAG AGC CTT CGG TCT GAA AGA AAC         481
Lys Lys Leu His Asp Arg Leu Arg Gln Ser Leu Arg Ser Glu Arg Asn
145                 150                 155                 160

ACC ATG ATG AAC GTA GCC AAC GGG CCC CAC CAC CCC AAT CCG CCC CCC         529
Thr Met Met Asn Val Ala Asn Gly Pro His His Pro Asn Pro Pro Pro
                165                 170                 175

GAG AAC GTG CAG CTG GTG AAT CAA TAC GTA TCT AAA AAT GTC ATC TCT         577
Glu Asn Val Gln Leu Val Asn Gln Tyr Val Ser Lys Asn Val Ile Ser
            180                 185                 190
```

Figure 16 B

```
AGC GAG CAT ATT GTT GAG AGA GAG GCG GAG AGC TCT TTT TCC ACC AGT          625
Ser Glu His Ile Val Glu Arg Glu Ala Glu Ser Ser Phe Ser Thr Ser
        195                 200                 205

CAC TAC ACT TCG ACA GCT CAT CAT TCC ACT ACT GTC ACT CAG ACT CCC          673
His Tyr Thr Ser Thr Ala His His Ser Thr Thr Val Thr Gln Thr Pro
    210                 215                 220

AGT CAC AGC TGG AGC AAT GGA CAC ACT GAA AGC ATC ATT TCG GAA AGC          721
Ser His Ser Trp Ser Asn Gly His Thr Glu Ser Ile Ile Ser Glu Ser
225                 230                 235                 240

CAC TCT GTC ATC GTG ATG TCA TCC GTA GAA AAC AGT AGG CAC AGC AGC          769
His Ser Val Ile Val Met Ser Ser Val Glu Asn Ser Arg His Ser Ser
                245                 250                 255

CCG ACT GGG GGC CCG AGA GGA CGT CTC AAT GGC TTG GGA GGC CCT CGT          817
Pro Thr Gly Gly Pro Arg Gly Arg Leu Asn Gly Leu Gly Gly Pro Arg
            260                 265                 270

GAA TGT AAC AGC TTC CTC AGG CAT GCC AGA GAA ACC CCT GAC TCC TAC          865
Glu Cys Asn Ser Phe Leu Arg His Ala Arg Glu Thr Pro Asp Ser Tyr
        275                 280                 285

CGA GAC TCT CCT CAT AGT GAA AGA CAT AAC CTT ATA GCT GAG CTA AGG          913
Arg Asp Ser Pro His Ser Glu Arg His Asn Leu Ile Ala Glu Leu Arg
    290                 295                 300

AGA AAC AAG GCC CAC AGA TCC AAA TGC ATG CAG ATC CAG CTT TCC GCA          961
Arg Asn Lys Ala His Arg Ser Lys Cys Met Gln Ile Gln Leu Ser Ala
305                 310                 315                 320

ACT CAT CTT AGA GCT TCT TCC ATT CCC CAT TGG GCT TCA TTC TCT AAG         1009
Thr His Leu Arg Ala Ser Ser Ile Pro His Trp Ala Ser Phe Ser Lys
                325                 330                 335

ACC CCT TGG CCT TTA GGA AGG TAT GTA TCA GCA ATG ACC ACC CCG GCT         1057
Thr Pro Trp Pro Leu Gly Arg Tyr Val Ser Ala Met Thr Thr Pro Ala
            340                 345                 350

CGT ATG TCA CCT GTA GAT TTC CAC ACG CCA AGC TCC CCC AAG TCA CCC         1105
Arg Met Ser Pro Val Asp Phe His Thr Pro Ser Ser Pro Lys Ser Pro
        355                 360                 365

CCT TCG GAA ATG TCC CCG CCC GTG TCC AGC ACG ACG GTC TCC ATG CCC         1153
Pro Ser Glu Met Ser Pro Pro Val Ser Ser Thr Thr Val Ser Met Pro
    370                 375                 380
```

Figure 16 C

```
TCC ATG GCG GTC AGT CCC TTC GTG GAA GAG GAG AGA CCC CTG CTC CTT          1201
Ser Met Ala Val Ser Pro Phe Val Glu Glu Glu Arg Pro Leu Leu Leu
385                 390                 395                 400

GTG ACG CCA CCA CGG CTG CGG GAG AAG TAT GAC CAC CAC GCC CAG CAA          1249
Val Thr Pro Pro Arg Leu Arg Glu Lys Tyr Asp His His Ala Gln Gln
                405                 410                 415

TTC AAC TCG TTC CAC TGC AAC CCC GCG CAT GAG AGC AAC AGC CTG CCC          1297
Phe Asn Ser Phe His Cys Asn Pro Ala His Glu Ser Asn Ser Leu Pro
            420                 425                 430

CCC AGC CCC TTG AGG ATA GTG GAG GAT GAG GAA TAT GAA ACG ACC CAG          1345
Pro Ser Pro Leu Arg Ile Val Glu Asp Glu Glu Tyr Glu Thr Thr Gln
        435                 440                 445

GAG TAC GAA CCA GCT CAA GAG CCG GTT AAG AAA CTC ACC AAC AGC AGC          1393
Glu Tyr Glu Pro Ala Gln Glu Pro Val Lys Lys Leu Thr Asn Ser Ser
    450                 455                 460

CGG CGG GCC AAA AGA ACC AAG CCC AAT GGT CAC ATT GCC CAC AGG TTG          1441
Arg Arg Ala Lys Arg Thr Lys Pro Asn Gly His Ile Ala His Arg Leu
465                 470                 475                 480

GAA ATG GAC AAC AAC ACA GGC GCT GAC AGC AGT AAC TCA GAG AGC GAA          1489
Glu Met Asp Asn Asn Thr Gly Ala Asp Ser Ser Asn Ser Glu Ser Glu
                485                 490                 495

ACA GAG GAT GAA AGA GTA GGA GAA GAT ACG CCT TTC CTG GCC ATA CAG          1537
Thr Glu Asp Glu Arg Val Gly Glu Asp Thr Pro Phe Leu Ala Ile Gln
            500                 505                 510

AAC CCC CTG GCA GCC AGT CTC GAG GCG GCC CCT GCC TTC CGC CTG GTC          1585
Asn Pro Leu Ala Ala Ser Leu Glu Ala Ala Pro Ala Phe Arg Leu Val
        515                 520                 525

GAC AGC AGG ACT AAC CCA ACA GGC GGC TTC TCT CCG CAG GAA GAA TTG          1633
Asp Ser Arg Thr Asn Pro Thr Gly Gly Phe Ser Pro Gln Glu Glu Leu
    530                 535                 540

CAG GCC AGG CTC TCC GGT GTA ATC GCT AAC CAA GAC CCT ATC GCT GTC          1681
Gln Ala Arg Leu Ser Gly Val Ile Ala Asn Gln Asp Pro Ile Ala Val
545                 550                 555                 560

TAAAACCGAA ATACACCCAT AGATTCACCT GTAAAACTTT ATTTTATATA ATAAAGTATT        1741

CCACCTTAAA TTAAACAAAA AAA                                               1764
```

Figure 17 A

F-B-A'	F-E-B-A'
F-B-A-C-C/D-D	F-E-B-A-C-C/D-D
F-B-A-C-C/D-H	F-E-B-A-C-C/D-H
F-B-A-C-C/D-H-L	F-E-B-A-C-C/D-H-L
F-B-A-C-C/D-H-K-L	F-E-B-A-C-C/D-H-K-L
F-B-A-C-C/D-D'-H	F-E-B-A-C-C/D-D'-H
F-B-A-C-C/D-D'-H-L	F-E-B-A-C-C/D-D'-H-L
F-B-A-C-C/D-D'-H-K-L	F-E-B-A-C-C/D-D'-H-K-L
F-B-A-C-C/D'-D	F-E-B-A-C-C/D'-D
F-B-A-C-C/D'-H	F-E-B-A-C-C/D'-H
F-B-A-C-C/D'-H-L	F-E-B-A-C-C/D'-H-L
F-B-A-C-C/D'-H-K-L	F-E-B-A-C-C/D'-H-K-L
F-B-A-C-C/D'-D'-H	F-E-B-A-C-C/D'-D'-H
F-B-A-C-C/D'-D'-H-L	F-E-B-A-C-C/D'-D'-H-L
F-B-A-C-C/D'-D'-'H-K-L	F-E-B-A-C-C/D'-D'-'H-K-L
F-B-A-C-C/D-C/D'-D	F-E-B-A-C-C/D-C/D'-D
F-B-A-C-C/D-C/D'-H	F-E-B-A-C-C/D-C/D'-H
F-B-A-C-C/D-C/D'-H-L	F-E-B-A-C-C/D-C/D'-H-L
F-B-A-C-C/D-C/D'-H-K-L	F-E-B-A-C-C/D-C/D'-H-K-L
F-B-A-C-C/D-C/D'-D'-H	F-E-B-A-C-C/D-C/D'-D'-H
F-B-A-C-C/D-C/D'-D'-H-L	F-E-B-A-C-C/D-C/D'-D'-H-L
F-B-A-C-C/D-C/D'-D'-H-K-L	F-E-B-A-C-C/D-C/D'-D'-H-K-L
F-B-A-G-C-C/D-D	F-E-B-A-G-C-C/D-D
F-B-A-G-C-C/D-H	F-E-B-A-G-C-C/D-H
F-B-A-G-C-C/D-H-L	F-E-B-A-G-C-C/D-H-L
F-B-A-G-C-C/D-H-K-L	F-E-B-A-G-C-C/D-H-K-L
F-B-A-G-C-C/D-D'-H	F-E-B-A-G-C-C/D-D'-H
F-B-A-G-C-C/D-D'-H-L	F-E-B-A-G-C-C/D-D'-H-L
F-B-A-G-C-C/D-D'-H-K-L	F-E-B-A-G-C-C/D-D'-H-K-L
F-B-A-G-C-C/D'-D	F-E-B-A-G-C-C/D'-D
F-B-A-G-C-C/D'-H	F-E-B-A-G-C-C/D'-H
F-B-A-G-C-C/D'-H-L	F-E-B-A-G-C-C/D'-H-L
F-B-A-G-C-C/D'-H-K-L	F-E-B-A-G-C-C/D'-H-K-L
F-B-A-G-C-C/D'-D'-H	F-E-B-A-G-C-C/D'-D'-H
F-B-A-G-C-C/D'-D'-H-L	F-E-B-A-G-C-C/D'-D'-H-L
F-B-A-G-C-C/D'-D'-'H-K-L	F-E-B-A-G-C-C/D'-D'-'H-K-L
F-B-A-G-C-C/D-C/D'-D	F-E-B-A-G-C-C/D-C/D'-D
F-B-A-G-C-C/D-C/D'-H	F-E-B-A-G-C-C/D-C/D'-H
F-B-A-G-C-C/D-C/D'-H-L	F-E-B-A-G-C-C/D-C/D'-H-L
F-B-A-G-C-C/D-C/D'-H-K-L	F-E-B-A-G-C-C/D-C/D'-H-K-L
F-B-A-G-C-C/D-C/D'-D'-H	F-E-B-A-G-C-C/D-C/D'-D'-H
F-B-A-G-C-C/D-C/D'-D'-H-L	F-E-B-A-G-C-C/D-C/D'-D'-H-L
F-B-A-G-C-C/D-C/D'-D'-H-K-L	F-E-B-A-G-C-C/D-C/D'-D'-H-K-L

Figure 17 B

E-B-A'

E-B-A-C-C/D-D
E-B-A-C-C/D-H
E-B-A-C-C/D-H-L
E-B-A-C-C/D-H-K-L
E-B-A-C-C/D-D'-H
E-B-A-C-C/D-D'-H-L
E-B-A-C-C/D-D'-H-K-L
E-B-A-C-C/D'-D
E-B-A-C-C/D'-H
E-B-A-C-C/D'-H-L
E-B-A-C-C/D'-H-K-L
E-B-A-C-C/D'-D'-H
E-B-A-C-C/D'-D'-H-L
E-B-A-C-C/D'-D'-H-K-L
E-B-A-C-C/D-C/D'-D
E-B-A-C-C/D-C/D'-H
E-B-A-C-C/D-C/D'-H-L
E-B-A-C-C/D-C/D'-H-K-L
E-B-A-C-C/D-C/D'-D'-H
E-B-A-C-C/D-C/D'-D'-H-L
E-B-A-C-C/D-C/D'-D'-H-K-L

E-B-A-G-C-C/D-D
E-B-A-G-C-C/D-H
E-B-A-G-C-C/D-H-L
E-B-A-G-C-C/D-H-K-L
E-B-A-G-C-C/D-D'-H
E-B-A-G-C-C/D-D'-H-L
E-B-A-G-C-C/D-D'-H-K-L
E-B-A-G-C-C/D'-D
E-B-A-G-C-C/D'-H
E-B-A-G-C-C/D'-H-L
E-B-A-G-C-C/D'-H-K-L
E-B-A-G-C-C/D'-D'-H
E-B-A-G-C-C/D'-D'-H-L
E-B-A-G-C-C/D'-D'-H-K-L
E-B-A-G-C-C/D-C/D'-D
E-B-A-G-C-C/D-C/D'-H
E-B-A-G-C-C/D-C/D'-H-L
E-B-A-G-C-C/D-C/D'-H-K-L
E-B-A-G-C-C/D-C/D'-D'-H
E-B-A-G-C-C/D-C/D'-D'-H-L
E-B-A-G-C-C/D-C/D'-D'-H-K-L

Figure 18

```
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT          48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1               5                   10                  15

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC          96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
            20                  25                  30

TTG TGC AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC         144
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
        35                  40                  45

GTA ATG GCC AGC TTC TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT         192
Val Met Ala Ser Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro
    50                  55                  60

GAA TAG                                                                 198
Glu
65
```

Figure 19

```
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT                48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1               5                   10                  15

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC                96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
            20                  25                  30

TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT               144
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn
        35                  40                  45

GTG CCC ATG AAA GTC CAA ACC CAA GAA AAA GCG GAG GAG CTC TAC TAA               192
Val Pro Met Lys Val Gln Thr Gln Glu Lys Ala Glu Glu Leu Tyr
    50                  55                  60
```

Figure 20

```
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT          48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1               5                   10                  15

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC          96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
            20                  25                  30

TTG TGC AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC         144
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
        35                  40                  45

GTA ATG GCC AGC TTC TAC AAA GCG GAG GAG CTC TAC TAA                     183
Val Met Ala Ser Phe Tyr Lys Ala Glu Glu Leu Tyr
    50                  55                  60
```

Figure 21

```
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT                48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1               5                   10                  15

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC                96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
            20                  25                  30

TTG TGC AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC               144
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
        35                  40                  45

GTA ATG GCC AGC TTC TAC AAG CAT CTT GGG ATT GAA TTT ATG GAG AAA               192
Val Met Ala Ser Phe Tyr Lys His Leu Gly Ile Glu Phe Met Glu Lys
    50                  55                  60

GCG GAG GAG CTC TAC TAA                                                      210
Ala Glu Glu Leu Tyr
65
```

Figure 22

```
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT          48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1               5                   10                  15

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC          96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
            20                  25                  30

TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT         144
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn
        35                  40                  45

GTG CCC ATG AAA GTC CAA ACC CAA GAA AAG TGC CCA AAT GAG TTT ACT         192
Val Pro Met Lys Val Gln Thr Gln Glu Lys Cys Pro Asn Glu Phe Thr
    50                  55                  60

GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC TAC AGT ACG TCC         240
Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe Tyr Ser Thr Ser
65                  70                  75                  80

ACT CCC TTT CTG TCT CTG CCT GAA TAG                                     267
Thr Pro Phe Leu Ser Leu Pro Glu
                85
```

Figure 23

```
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT                48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1               5                   10                  15

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC                96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
            20                  25                  30

TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT               144
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn
        35                  40                  45

GTG CCC ATG AAA GTC CAA ACC CAA GAA AAG TGC CCA AAT GAG TTT ACT               192
Val Pro Met Lys Val Gln Thr Gln Glu Lys Cys Pro Asn Glu Phe Thr
    50                  55                  60

GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC TAC AAA GCG GAG               240
Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe Tyr Lys Ala Glu
65                  70                  75                  80

GAG CTC TAC TAA                                                               252
Glu Leu Tyr
```

Figure 24 A

```
GGAATTCCTT TTTTTTTTTT TTTTTTTCTT NNTTTTTTTT TGCCCTTATA CCTCTTCGCC          60

TTTCTGTGGT TCCATCCACT TCTTCCCCCT CCTCCTCCCA TAAACAACTC TCCTACCCCT         120

GCACCCCCAA TAAATAAATA AAAGGAGGAG GGCAAGGGGG GAGGAGGAGG AGTGGTGCTG         180

CGAGGGGAAG GAAAAGGGAG GCAGCGCGAG AAGAGCCGGG CAGAGTCCGA ACCGACAGCC         240

AGAAGCCCGC ACGCACCTCG CACC ATG AGA TGG CGA CGC GCC CCG CGC CGC              291
                           Met Arg Trp Arg Arg Ala Pro Arg Arg
                            1               5

TCC GGG CGT CCC GGC CCC CGG GCC CAG CGC CCC GGC TCC GCC GCC CGC            339
Ser Gly Arg Pro Gly Pro Arg Ala Gln Arg Pro Gly Ser Ala Ala Arg
 10                  15                  20                  25

TCG TCG CCG CCG CTG CCG CTG CTG CCA CTA CTG CTG CTG CTG GGG ACC            387
Ser Ser Pro Pro Leu Pro Leu Leu Pro Leu Leu Leu Leu Leu Gly Thr
                                        Val Cys Leu Leu Thr Val
                                                    GGF II 09
                30                  35                  40

GCG GCC CTG GCG CCG GGG GCG GCG GCC GGC AAC GAG GCG GCT CCC GCG            435
Ala Ala Leu Ala Pro Gly Ala Ala Ala Gly Asn Glu Ala Ala Pro Ala
Ala Ala Leu Pro Pro
            45                  50                  55

GGG GCC TCG GTG TGC TAC TCG TCC CCG CCC AGC GTG GGA TCG GTG CAG            483
Gly Ala Ser Val Cys Tyr Ser Ser Pro Pro Ser Val Gly Ser Val Gln
                         Ala Ser Pro Val Ser Val Gly Ser Val Gln
                                                GGF II 08
        60                  65                  70

GAG CTA GCT CAG CGC | GCC GCG GTG GTG ATC GAG GGA AAG GTG CAC CCG          531
Glu Leu Ala Gln Arg | Ala Ala Val Val Ile Glu Gly Lys Val His Pro
Glu Leu Val Gln Arg | Trp Phe Val Val Ile Glu Gly Lys
                              GGF II 04
    75                  80                  85
```

Figure 24 B

```
CAG CGG CGG CAG CAG GGG GCA CTC GAC AGG AAG GCG GCG GCG GCG GCG          579
Gln Arg Arg Gln Gln Gly Ala Leu Asp Arg Lys Ala Ala Ala Ala Ala
 90                  95                  100                 105

GGC GAG GCA GGG GCG TGG GGC GGC GAT CGC GAG CCG CCA GCC GCG GGC          627
Gly Glu Ala Gly Ala Trp Gly Gly Asp Arg Glu Pro Pro Ala Ala Gly
                110                 115                 120

CCA CGG GCG CTG GGG CCG CCC GCC GAG GAG CCG CTG CTC GCC GCC AAC          675
Pro Arg Ala Leu Gly Pro Pro Ala Glu Glu Pro Leu Leu Ala Ala Asn
            125                 130                 135

GGG ACC GTG CCC TCT TGG CCC ACC GCC CCG GTG CCC AGC GCC GGC GAG          723
Gly Thr Val Pro Ser Trp Pro Thr Ala Pro Val Pro Ser Ala Gly Glu
        140                 145                 150

CCC GGG GAG GAG GCG CCC TAT CTG GTG AAG GTG CAC CAG GTG TGG GCG          771
Pro Gly Glu Glu Ala Pro Tyr Leu Val Lys Val His Gln Val Trp Ala
                                    Lys Val His Glu Val Trp Ala
                                          GGF II 01 & GGF II 11
    155                 160                 165

GTG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG CTC ACC GTG CGC CTG          819
Val Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu Leu Thr Val Arg Leu
Ala Lys                             Asp Leu Leu Leu Xaa Val     Leu
                                          GGF II 10
170                 175                 180                 185

GGG ACC TGG GGC CAC CCC GCC TTC CCC TCC TGC GGG AGG CTC AAG GAG          867
Gly Thr Trp Gly His Pro Ala Phe Pro Ser Cys Gly Arg Leu Lys Glu
Gly Ala Trp Gly Pro Pro Ala Phe Pro Val Xaa Tyr
               GGF II 03
                190                 195                 200

GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAC GCC AAC AGC ACC AGC          915
Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro Asp Ala Asn Ser Thr Ser
            Tyr Ile Phe Phe Met Glu Pro Asp Ala Xaa Ser Ser Gly
                                    GGF II 02
            205                 210                 215
```

Figure 24 C

```
CGC GCG CCG GCC GCC TTC CGA GCC TCT TTC CCC CCT CTG GAG ACG GGC          963
Arg Ala Pro Ala Ala Phe Arg Ala Ser Phe Pro Pro Leu Glu Thr Gly
        220                 225                 230

CGG AAC CTC AAG AAG GAG GTC AGC CGG GTG CTG TGC AAG CGG TGC GCC         1011
Arg Asn Leu Lys Lys Glu Val Ser Arg Val Leu Cys Lys Arg Cys Ala
    235                 240                 245

TTG CCT CCC CAA TTG AAA GAG ATG AAA AGC CAG GAA TCG GCT GCA GGT         1059
Leu Pro Pro Gln Leu Lys Glu Met Lys Ser Gln Glu Ser Ala Ala Gly
250                 255                 260                 265

TCC AAA CTA GTC CTT CGG TGT GAA ACC AGT TCT GAA TAC TCC TCT CTC         1107
Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser Ser Leu
        Leu Val Leu Arg
            GGF II 06
            270                 175                 180

AGA TTC AAG TGG TTC AAG AAT GGG AAT GAA TTG AAT CGA AAA AAC AAA         1155
Arg Phe Lys Trp Phe Lys Asn Gly Asn Glu Leu Asn Arg Lys Asn Lys
        185                 190                 195

CCA CAA AAT ATC AAG ATA CAA AAA AAG CCA GGG AAG TCA GAA CTT CGC         1203
Pro Gln Asn Ile Lys Ile Gln Lys Lys Pro Gly Lys Ser Glu Leu Arg
        200                 205                 210

ATT AAC AAA GCA TCA CTG GCT GAT TCT GGA GAG TAT ATG TGC AAA GTG         1251
Ile Asn Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr Met Cys Lys Val
        Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr Met Xaa Lys
                                GGF II 12
    215                 220                 225

ATC AGC AAA TTA GGA AAT GAC AGT GCC TCT GCC AAT ATC ACC ATC GTG         1299
Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn Ile Thr Ile Val
230                 235                 240                 245

GAA TCA AAC GCT ACA TCT ACA TCC ACC ACT GGG ACA AGC CAT CTT GTA         1347
Glu Ser Asn Ala Thr Ser Thr Ser Thr Thr Gly Thr Ser His Leu Val
            250                 255                 260
```

Figure 24 D

```
AAA TGT GCG GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGG GAG TGC          1395
Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys
            265                 270                 275

TTC ATG GTG AAA GAC CTT TCA AAC CCC TCG AGA TAC TTG TGC AAG TGC          1443
Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr Leu Cys Lys Cys
        280                 285                 290

CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC          1491
Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser
    295                 300                 305

TTC TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA                      1530
Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro Glu
400                 405                 410

TAGGAGCATG CTCAGTTGGT GCTGCTTTCT TGTTGCTGCA TCTCCCCTCA GATTCCACCT        1590

AGAGCTAGAT GTGTCTTACC AGATCTAATA TTGACTGCCT CTGCCTGTCG CATGAGAACA        1650

TTAACAAAAG CAATTGTATT ACTTCCTCTG TTCGCGACTA GTTGGCTCTG AGATACTAAT        1710

AGGTGTGTGA GGCTCCGGAT GTTTCTGGAA TTGATATTGA ATGATGTGAT ACAAATTGAT        1770

AGTCAATATC AAGCAGTGAA ATATGATAAT AAAGGCATTT CAAAGTCTCA CTTTTATTGA        1830

TAAAATAAAA ATCATTCTAC TGAACAGTCC ATCTTCTTTA TACAATGACC ACATCCTGAA        1890

AAGGGTGTTG CTAAGCTGTA ACCGATATGC ACTTGAAATG ATGGTAAGTT AATTTTGATT        1950

CAGAATGTGT TATTTGTCAC AAATAAACAT AATAAAAGGA AAAAAAAAAA AAA               2003
```

Figure 25

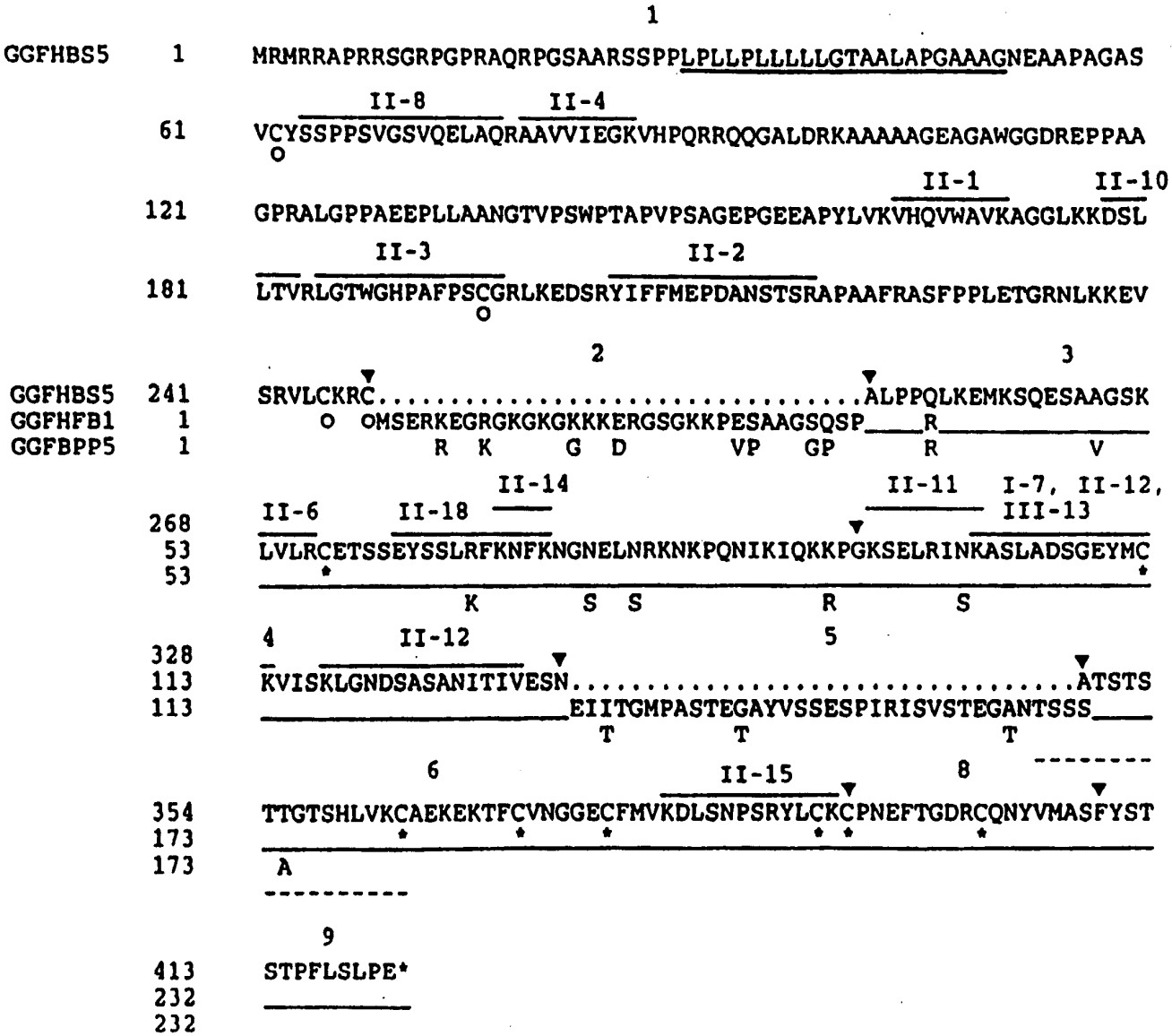
1
GGFHBS5 1 MRMRRAPRRSGRPGPRAQRPGSAARSSPPLPLLPLLLLLGTAALAPGAAAGNEAAPAGAS
II-8
II-4
61 VCYSSPPSVGSVQELAQRAAVVIEGKVHPQRRQQGALDRKAAAAAGEAGAWGGDREPPAA
II-1
II-10
121 GPRALGPPAEEPLLAANGTVPSWPTAPVPSAGEPGEEAPYLVKVHQVWAVKAGGLKKDSL
II-3
II-2
181 LTVRLGTWGHPAFPSCGRLKEDSRYIFFMEPDANSTSRAPAAFRASFPPLETGRNLKKEV
2
3
GGFHBS5 241 SRVLCKRC.....ALPPQLKEMKSQESAAGSK
GGFHFB1 1 OMSERKEGRGKGKGKKKERGSGKKPESAAGSQSP____R_____
GGFBPP5 1 R K G D VP GP R V
II-14
II-11
I-7, II-12,
II-6
II-18
III-13
268 LVLRCETSSEYSSLRFKNFKNGNELNRKNKPQNIKIQKKPGKSELRINKASLADSGEYMC
53
53
K S S R S
4
II-12
5
328
113 KVISKLGNDSASANITIVESN.....ATSTS
113 _____EIITGMPASTEGAYVSSESPIRISVSTEGANTSSS____
T T T
6
II-15
8
354 TTGTSHLVKCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCPNEFTGDRCQNYVMASFYST
173
173
A
9
413 STPFLSLPE*
232
232

Figure 26

```
Met Arg Trp Arg Arg Ala Pro Arg Arg Ser Gly Arg Pro Gly Pro Arg
 1               5                  10                  15

Ala Gln Arg Pro Gly Ser Ala Ala Arg Ser Ser Pro Pro Leu Pro Leu
            20                  25                  30

Leu Pro Leu Leu Leu Leu Leu Gly Thr Ala Ala Leu Ala Pro Gly Ala
        35                  40                  45

Ala Ala Gly Asn Glu Ala Ala Pro Ala Gly Ala Ser Val Cys Tyr Ser
    50                  55                  60

Ser Pro Pro Ser Val Gly Ser Val Gln Glu Leu Ala Gln Arg Ala Ala
65                  70                  75                  80

Val Val Ile Glu Gly Lys Val His Pro Gln Arg Arg Gln Gln Gly Ala
                85                  90                  95

Leu Asp Arg Lys Ala Ala Ala Ala Ala Gly Glu Ala Gly Ala Trp Gly
            100                 105                 110

Gly Asp Arg Glu Pro Pro Ala Ala Gly Pro Arg Ala Leu Gly Pro Pro
        115                 120                 125

Ala Glu Glu Pro Leu Leu Ala Ala Asn Gly Thr Val Pro Ser Trp Pro
    130                 135                 140

Thr Ala Pro Val Pro Ser Ala Gly Glu Pro Gly Glu Glu Ala Pro Tyr
145                 150                 155                 160

Leu Val Lys Val His Gln Val Trp Ala Val Lys Ala Gly Gly Leu Lys
                165                 170                 175

Lys Asp Ser Leu Leu Thr Val Arg Leu Gly Thr Trp Gly His Pro Ala
            180                 185                 190

Phe Pro Ser Cys Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe
        195                 200                 205

Met Glu Pro Asp Ala Asn Ser Thr Ser Arg Ala Pro Ala Ala Phe Arg
    210                 215                 220

Ala Ser Phe Pro Pro Leu Glu Thr Gly Arg Asn Leu Lys Lys Glu Val
225                 230                 235                 240

Ser Arg Val Leu Cys Lys Arg Cys Ala Leu Pro Pro Gln Leu Lys Glu
                245                 250                 255

Met Lys Ser Gln Glu Ser Ala Ala Gly Ser Lys Leu Val Leu Arg Cys
            260                 265                 270

Glu Thr Ser Ser Glu Tyr Ser Ser Leu Arg Phe Lys Trp Phe Lys Asn
        275                 280                 285

Gly Asn Glu Leu Asn Arg Lys Asn Lys Pro Gln Asn Ile Lys Ile Gln
    290                 295                 300

Lys Lys Pro Gly Lys Ser Glu Leu Arg Ile Asn Lys Ala Ser Leu Ala
305                 310                 315                 320

Asp Ser Gly Glu Tyr Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp
                325                 330                 335
```

Figure 26 (cont.)

```
Ser Ala Ser Ala Asn Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr
           340                 345                 350

Ser Thr Thr Gly Thr Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys
       355                 360                 365

Thr Phe Cys Val Asn Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser
   370                 375                 380

Asn Pro Ser Arg Tyr Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp
385                 390                 395                 400

Arg Cys Gln Asn Tyr Val Met Ala Ser Phe Tyr Ser Thr Ser Thr Pro
                405                 410                 415

Phe Leu Ser Leu Pro Glu *
           420
```

Figure 27

```
TCTAA AAC TAC AGA GAC TGT ATT TTC ATG ATC ATC ATA GTT CTG TGA AAT ATA      53
      Asn Tyr Arg Asp Cys Ile Phe Met Ile Ile Ile Val Leu Xaa Asn Ile
        1               5                  10                  15

CTT AAA CCG CTT TGG TCC TGA TCT TGT AGG AAG TCA GAA CTT CGC ATT          101
Leu Lys Pro Leu Trp Ser Xaa Ser Cys Arg Lys Ser Glu Leu Arg Ile
            20                  25                  30

AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT ATG TGC AAA GTG ATC          149
Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Ser Met Cys Lys Val Ile
        35                  40                  45

AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC ATC ACC ATT GTG GAG          197
Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn Ile Arg Ile Val Glu
    50                  55                  60

TCA AAC GGT AAG AGA TGC CTA CTG CGT GCT ATT TCT CAG TCT CTA AGA          245
Ser Asn Gly Lys Arg Cys Leu Leu Arg Ala Ile Ser Gln Ser Leu Arg
65                  70                  75                  80

GGA GTG ATC AAG GTA TGT GGT CAC ACT TGA ATC ACG CAG GTG TGT GAA          293
Gly Val Ile Lys Val Cys Gly His Thr Xaa Ile Thr Gln Val Cys Glu
                85                  90                  95

ATC TCA TTG TGA ACA AAT AAA AAT CAT GAA AGG AAA ACT CTA TGT TTG.         341
Ile Ser Cys Xaa Thr Asn Lys Asn His Glu Arg Lys Thr Leu Cys Leu
            100                 105                 110

AAA TAT CTT ATG GGT CCT CCT GTA AAG CTC TTC ACT CCA TAA GGT GAA          389
Lys Tyr Leu Met Gly Pro Pro Val Lys Leu Phe Thr Pro Xaa Gly Glu
        115                 120                 125

ATA GAC CTG AAA TAT ATA TAG ATT ATT T                                    417
Ile Asp Leu Lys Tyr Ile Xaa Ile Ile
    130                 135
```